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Influence of rice straw-derived dissolved organic matter on lactic acid fermentation by *Rhizopus oryzae*

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Rice straw can be used as carbon sources for lactic acid fermentation. However, only a small amount of lactic acid is produced even though *Rhizopus oryzae* can consume glucose in rice straw-derived hydrolysates. This study correlated the inhibitory effect of rice straw with rice straw-derived dissolved organic matter (DOM). Lactic acid fermentations with and without DOM were conducted to investigate the effect of DOM on lactic acid fermentation by *R. oryzae*. Fermentation using control medium with DOM showed a similar trend to fermentation with rice straw-derived hydrolysates, showing that DOM contained the major inhibitor of rice straw. DOM assay indicated that it mainly consisted of polyphenols and polysaccharides. The addition of polyphenols and polysaccharides derived from rice straw confirmed that lactic acid fermentation was promoted by polysaccharides and significantly inhibited by polyphenols. The removal of polyphenols also improved lactic acid production. However, the loss of polysaccharides during the removal of polyphenols resulted in low glucose consumption. This study is the first to investigate the effects of rice straw-derived DOM on lactic acid fermentation by *R. oryzae*. The results may provide a theoretical basis for identifying inhibitors and promoters associated with lactic acid fermentation and for establishing suitable pretreatment methods.

[**Key words:** Lactic acid fermentation; *Rhizopus oryzae*; Polyphenols; Polysaccharides; Rice straw]

Lactic acid is widely used in the food, brewing, and medical industries; in particular, it is utilized as raw material for biodegradable and renewable plastics (1). Lactic acid production from crops (2), such as sugarcane and corn, by using limited land resources, can potentially cause a biomass deficit (3). Lignocellulosic materials, such as forestry and agricultural residues, could be used as alternative feedstock for lactic acid production without affecting the food supply (4). Therefore, studies have focused on the bioconversion of abundant and renewable lignocellulosic biomass to lactic acid (5,6). A fungal species, *Rhizopus oryzae*, can convert glucose, xylose, galactose, and starch into lactic acid (7). Recently, many studies have reported lactic acid was successfully produced through fermentation using *R. oryzae* (8,9). Since *R. oryzae* exhibits amylase and endo-beta-glucanase, it also shows the potential for bioconversion of agricultural wastes (10). Dissolved organic matter (DOM) is a heterogeneous mixture of different organic compounds primarily derived from plant and microbial residues (11). DOM is a highly active organic component in terrestrial ecosystems and contains low molecular weight compounds (e.g., free amino acids, sugars, and organic acids) and high molecular weight compounds (e.g., humic substances, amino sugars, and polyphenols) (12).

Rice straw is a major lignocellulosic residue produced worldwide and the estimated amount of rice straw available is 685 million tons per year (13). Rice straw-derived DOM is often used as soil conditioner for soil remediation (14). In contrast to herbaceous materials, such as clover residues, straw residues contain lower levels of easily utilizable sugars and proteins and higher amounts of celluloses and hemicelluloses (15,16). Straw-derived DOM contains large amounts of carbohydrates and aromatic compounds. During straw decay, the DOM content in the soil markedly increases and the dissolution of soil organic matter is enhanced (17). Chen et al. (18) reported that various DOM species decomposed at different stages by microorganisms were derived from straw decay.

Decay of plant straw in soil is a slow process. In contrast, decomposition of straw is a rapid process during bioconversion of rice straw to lactic acid because pretreatment with the use of cellulase in hydrolysis releases large amounts of DOM. However, the mechanism through which DOM affects lactic acid fermentation remains unclear. Studies on fermentation inhibitors have generally been limited to substances produced by destruction of straw components, such as 5-hydroxymethylfurfural and furfural (19,20), during pretreatment. Moreover, the inhibitory effects of substances released from the straw itself have not been investigated yet. To develop efficient pretreatment processes for lactic acid production, the release mechanism of DOM from rice straw and its effect on fermentation should be identified. This study aims to (i)

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determine the composition of DOM released from rice straw, (ii) investigate the inhibitory effect of DOM on lactic acid fermentation by *R. oryzae*, (iii) characterize the components of DOM that act as inhibitors, and (iv) evaluate the effects of inhibitor removal on lactic acid fermentation by *R. oryzae*.

MATERIALS AND METHODS

Rice straw Rice straw was obtained from several fields on Chongming Island, Shanghai, China. The collected straw samples were air-dried, ground, and passed through a 1-mm aperture standard screen. The samples were then kept in an oven at 45°C prior to pretreatment (21).

Preparation of DOM Ten grams of the processed rice straw was milled at 400 rpm with a Desk-Top Planetary Miller (SFM-1, Kejing Materials Technology Co., Shanghai, China) at room temperature for 15 cycles, using a jar of 0.5 L with 1/3 of balls (diameter of 0.5 cm). A cycle mode of 10-min milling followed by 5-min pause was adopted (3). The ball-milled straw was extracted with water, 1% NaOH or 1% H_2SO_4 (w/w), in a solid-to-liquid ratio of 1:10 at 60°C and 150 rpm for 60 min. DOM was prepared by centrifuging the mixtures at 8000 ×*g* for 10 min. The DOM pretreated with hot water was used to investigate the effects of DOM on lactic acid fermentation by *R. oryzae*.

Preparation of polysaccharides and polyphenols The sample was extracted with water in a solid-to-liquid ratio of 1:10 (w/v) at 60°C and 150 rpm for 60 min. The extract was added with ethanol to a concentration of 70%. The sediment was used as crude polysaccharide fraction (22). The supernatant was then collected by filtration and evaporated to remove ethanol. The concentrated supernatant with water resolution was used as crude polyphenol fraction.

Removal of polyphenols from rice straw Ten grams of the milled straw was added to 1% NaOH or 1% H_2SO_4 (w/v) in a 250-mL flask in a solid-to-liquid ratio of 1:10 (w/v) and then shaken at 60°C and 150 rpm for 60 min. After centrifugation at 8000 ×*g* for 10 min, the solid fraction was washed with 100 mL of water three times neutralized with HCl or NaOH. The treated solid was the used for enzymatic hydrolysis of rice straw. The content of polyphenols in the hydrolysates was then determined.

Fungus culture and control fermentation *R. oryzae* strain (CICC M198) was purchased from Shanghai Industrial Microbiology Institute Tech. Co., Ltd. (Shanghai, China). The spores collected from a PDA slant were suspended in sterilized water and then directly added to the fermentation medium to obtain a spore count of 1×10^5 per milliliter. The number of spores was counted with a hemacytometer. The control fermentation was conducted at 37°C and 150 rpm with a medium containing the following (g L^{-1}): 50 glucose, 0.125 $(NH_4)_2SO_4$, 0.6 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$, 30 $CaCO_3$, and 2.0 yeast extract. The inhibitory effects of DOM were investigated by the fermentations using media with DOM. Fermentations using commercial tannin, phenolic acids (Sinopharm, Shanghai, China) and two polysaccharides (i.e., xylan from beechwood or corn cob) (Sigma-Aldrich, St. Louis, MO, USA) were served as control.

Analysis of phenolic compounds The content of total phenolics (TP) was measured through colorimetric method using Folin-Ciocalteu reagent method with minor modification (23). Approximately 1 mL of the diluted sample (20 ×) was mixed with 5.5 mL of distilled water and 1.5 mL of Folin phenol solution for 5 min, and then sodium bicarbonate (10% w/v) was added along with 2 mL of distilled water. The mixture was stored in the dark at ambient temperature for 4 h. The absorbance of the mixture was recorded using UV−vis spectrophotometer (Unico UV-2102C, Shanghai, China) at 765 nm. TP content was quantified based on the standard curve of gallic acid prepared in 80% methanol (v/v). The phenolic acids were evaluated using the same procedure and equipment as described elsewhere (13).

Tannin in each fraction was quantified using a modified vanillin assay (24). A 2.5-mL aliquot of 1:3 (v/v) sulfuric acid/methanol solutions and 2.5-mL of 1% (w/v) vanillin in methanol solution were mixed with 1 mL of the sample. The tubes were incubated at 30°C for 12 h. Absorbance of each tube was recorded 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 each corresponding vanillin-containing sample. The value was compared with the standard curve for catechin. The concentration of condensed tannin was expressed as gram of catechin per liter.

Analysis of carbohydrate compositions of soluble polysaccharides The carbohydrate composition of soluble polysaccharides was measured using the previously described acid hydrolysis method (25). The freeze-dried solid was acid-hydrolyzed with 72% (v/v) sulfuric acid at 30°C for 1 h, followed by acid hydrolysis with 4% (w/w) sulfuric acid at 121°C for 1 h. After hydrolysis, the hydrolysate was filtered for HPLC analysis.

Analysis of glucose, lactic acid, and ethanol Glucose, lactic acid, and ethanol were analyzed using HPLC (LC-20AD, Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65°C. 5 mM H_2SO_4 was used for the mobile phase at a flow rate of 0.6 mL/min. All experiments were performed in triplicate and the averages of data were reported (3).

TABLE 1. Components of different rice straw-derived DOM (g/100 g straw).

	TOC	Polysaccharides	Total phenolics	Tannins
Water	4.96 ± 0.06	4.42 ± 0.12	0.47 ± 0.03	0.20 ± 0.01
1% NaOH	13.09 ± 0.30	26.74 ± 1.16	2.15 ± 0.02	0.23 ± 0.02
1% H_2SO_4	4.75 ± 0.03	10.67 ± 0.22	0.36 ± 0.02	0.05 ± 0.00

The DOM was prepared with hot water, 1% NaOH or 1% H_2SO_4 at 60°C and 150 rpm for 60 min. The data represent averages ± standard deviations for triplicate experiments.

Analysis of total organic carbon The supernatant was filtered using 4.5 μm membrane filters, and the total organic carbon (TOC) value was analyzed using a Shimadzu TOC-VCPH total organic carbon analyzer (Shimadzu). To reduce the impact of inorganic carbon on the analyses, the pH of the sample was adjusted to about 4.0 prior to TOC analysis.

RESULTS AND DISCUSSION

Assays of DOM DOM is a heterogeneous mixture containing various organic substances, particularly low-molecular-weight compounds (e.g., free amino acids, sugars, and organic acids) and high-molecular-weight compounds (e.g., humic substances, amino sugars, and polyphenols) (12). Rice straw is a typical lignocellulosic biomass, as such, the components of rice straw-derived DOM were determined based on polyphenol and polysaccharide contents. Table 1 shows that the components of DOM mainly contained polysaccharides and polyphenols. The results indicated pretreatments played important roles in determining the components of DOM. The TOC yield of DOM was 4.96 g/100 g when rice straw was extracted with water. The NaOH pretreatment significantly increased the TOC yield of DOM, which could be attributed to the increase in the total phenolics and polysaccharides. The yields of polysaccharide and total phenolics increased to 26.74 g/100 g and 2.15 g/100 g, which are 6.0- and 4.6-fold higher than that in water-based extraction, respectively. The yield of tannin was 0.23 g/100 g, which is close to the value obtained from water-based extraction. However, H_2SO_4 pretreatment did not affect the yield of TOC, despite that large amounts of polysaccharides were released under the said condition. This finding could be due to the destruction of organic matter by the acid.

Plant polyphenols are secondary metabolites and can be categorized into: (i) polyphenol monomers, including flavonoid compounds, chlorogenic acids, gallic acid and ellagic acid, and (ii) tannin substances produced through polymerization of monomers and oligomers or polymers, such as proanthocyanidins and gallotannins (26). Most phenolic acids are ether-linked to lignin and polysaccharides (27), and the linked phenolic acids cannot be released with water (28). However, tannin was detected in the DOM. In this study, tannin was mainly used for the assay of the oligomers of polyphenols because they are often found in the layer between epidermis and cortex; hence, extraction with hot water can easily release tannin from rice straw (29). Tannin toxicity to rumen microorganisms has been reported and three toxicity mechanisms have been identified, namely enzyme inhibition and substrate deprivation, membrane action, and metal ion deprivation in recent studies. As shown in Table 1, 0.20 g/100 g tannin were released through water-based extraction, whereas 0.23 and 0.05 g/100 g tannin were released through extractions with 1% NaOH and H_2SO_4 (w/w), respectively. These results indicate that acid pretreatment could effectively destroy tannin. To present the influence of the polyphenols, DOM from water-based extraction was used as control to investigate the effect of DOM on lactic acid fermentation in this study. The inhibitory effects of rice straw-derived DOM

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