

Supplementary materials for the article:

Xiong Y. et al. Characterization of Ligninolytic Bacteria and Analysis of Alkali-Lignin Biodegradation Products
Pol J Microbiol. 2020, Vol. 69, No 3, 339–347.

Methods and Materials

Screening and identification of ligninolytic bacteria. The ligninolytic bacteria in this experiment mainly isolated from soil, straw and silage. The humus-rich soil samples were collected from the campus of Shanxi Agricultural University. The straw compost (no additive was added) was collected from a harvested cornfield in Jinzhong, Shanxi Province, and the silage samples were taken from a husbandry cooperative which made whole plant corn silage (fermented without additive) in Taigu, Shanxi Province. The samples (500 g) were taken and placed in a sample box (ESKY, 12 l) containing an icepack and brought back to the laboratory.

10 g of each sample were placed in a sterile Erlenmeyer flask, and 50 ml of 0.9% physiological saline was added, then stirred it violently by a vortex mixer. Diluted mixed liquids by five levels (1×10^{-1} , 1×10^{-2} , 1×10^{-3} , 1×10^{-4} , 1×10^{-5}) and inoculated 100 μ l into the ligninolytic selection medium. The medium contained 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g MgSO_4 , 1.0 g K_2HPO_4 , 0.5 g NaCl, 5.0 g alkaline lignin, 20.0 g Agar powder, 1.0 l H_2O . All the chemicals were purchased from Solaibao Biotechnology Co., Ltd. In the ultra-clean bench, coated it with a triangular glass rod, and finally the plate was inverted and cultured at 30°C for 48 h. Finally, single colonies were inoculated into LB tube medium.

The decolorization of aniline blue proved that bacteria could produce ligninolytic enzymes. Also the decolorization of brilliant blue was considered to that laccase were produced in the plate. therefore, 1% aniline blue or brilliant blue was added into the alkaline lignin medium by a sterile filter (0.45 μ m), respectively. Afterward, the bacteria which had the hydrolysis circle were selected and purified. Finally, they were stored at -20°C with liquid paraffin.

The total DNA of the strain was extracted using the Omega Bacterial Genomic DNA Extraction Kit, and then the 16S rDNA primer sequence of the bacterial PCR was amplified by the 27F and 1492R primers as follows: 27F: (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R: (5'-TACGGCTACCTTGTTACGACTT-3'). The bacteria were sequenced by Nanchang Kechang Biotechnology company.

The 16S rDNA sequence of the identified strain was imported into MEGA 7.0 for phylogenetic analysis, and the phylogenetic tree was constructed by Neighbor-join method.

Characterization of ligninolytic bacteria. 2 ml of the fermentation broth was added to the centrifuge tube, centrifuged at 8,000 g for 3 min at 4°C, the supernatant liquid was discarded, the precipitate was washed by adding a phosphate buffer solution (pH 7.2), and then centrifuged again. After repeating 3 times, the supernatant was discarded. 2.5% glutaraldehyde was added and fixed in a refrigerator at 4°C for 24 h. The ethanol was used for

the gradient (30%, 50%, 70%, 80%, 90%) dehydration treatment. After gradient dehydration centrifugation, it was eluted twice with absolute ethanol, and the supernatant was discarded by centrifugation, and the bacteria were resuspended in absolute ethanol. The coverslips were immersed in 1 M HCl solution for 12 h, and the coverslips were washed with absolute ethanol, sonicated for 30 min and dried. 5–10 μ l of the bacterial liquid after resuspending was pipetted and added to cover glass. After drying, the sample was observed by scanning electron microscopy.

Lip activity was detected by lignin peroxidase kit (Beijing Solabao Technology Co., Ltd.). Lip oxidized resveratrol to produce veratraldehyde with a specific absorption peak at 310 nm (Yang et al. 2017; Zhou et al. 2017). The bacterial cultural solution was centrifuged at 10,000 g for 10 min, and the supernatant was placed in a 2 ml centrifuge tube and placed on ice for testing. The reaction system contained 1 mM resveratrol, 50 mM phosphate buffer (PBS), pH 7.2, and 0.1 mM hydrogen peroxide, and the supernatant was added in an amount of 100 μ l. Ultrapure water was used as a control to measure the absorbance of 10 S and 310 S at 310 nm, which were recorded as A1 and A2, and $\Delta A = A2 - A1$. The enzyme activity is defined as the amount of enzyme required to oxidize 1 nmol of resveratrol per liter of culture solution is one enzyme activity unit, and the molar extinction coefficient of veratraldehyde, $\epsilon_1 = 9,300 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Mnp is also an oxidase containing heme, which is oxidized with guaiacol to tetra-o-methoxyphenol in the presence of Mn^{2+} , and has a characteristic absorption peak at 465 nm (Hwang et al. 2008). Mnp activity was detected by manganese peroxidase kit (Beijing Suo Laibao Technology Co., Ltd.). The culture solution was centrifuged at 10,000 g for 10 min, and the supernatant was placed on ice to be tested as a crude enzyme solution. A 100 μ l sample and 900 μ l of the substrate were thoroughly mixed in a 1 ml glass cuvette as a reaction system. After reacting at 37°C for 10 min, and ultrapure water as a control, the absorbance at 465 nm was measured to calculate the difference ΔA . Enzyme activity definition: The amount of enzyme required to oxidize 1 nmol of guaiacol per minute per liter of the culture medium is an enzyme activity unit. The guaiacol extinction coefficient $\epsilon_2: 12,100 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, the calculation formula is as follows:

$$\text{Lip}(\text{nmol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}) = \Delta A \div (\epsilon_1 \times d) \times V_A \div V_S \div T$$

$$\text{Mnp}(\text{nmol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}) = \Delta A \div (\epsilon_2 \times d) \times V_A \div V_S \div T$$

d – cuvette light path, V_A – total reaction volume, V_S – sample volume in the reaction, T – reaction time.

Degradation product analysis. Strains were inoculated in a liquid medium and placed on a constant temperature shaker, and they were cultured for 72 h, respectively. The fermentation broth of different strains was centrifuged for 5 min at a temperature of 4°C and a centrifugal force of 10,000 g. After centrifugation, the supernatant was placed in a new centrifuge tube for measurement.

The pH meter was calibrated and the pH of the supernatant was determined and recorded. The pH of the supernatant was adjusted to about 2.0 with 38% concentrated hydrochloric acid. After adjusting the pH, the fermentation broth was mixed and then extracted using accelerated solvent extraction (ASE300). 1 ml of liquid was solidified by using diatomaceous. In the extraction vessel, ethyl acetate was used as an extractant, and after extraction, ethyl acetate was blown off using a nitrogen purifier to obtain an extracted

product. After nitrogen drying, the product was dissolved in 100 μl of cyclohexane, dioxane and ethyl acetate, respectively. At the same time, 50 μl of a trimethylsilyl trifluoroacetamide (BSTFA (N,O-bis (trimethylsilyl))) was added to facilitate determination.

The dissolved sample was analyzed by GC-MS. The liquid was injected using Thermo Trace 1300 ISQ, the injection volume was 1 μl , the column was OM-5MS capillary column, the carrier gas was He gas, and the flow rate was controlled at 1 $\text{ml} \cdot \text{min}^{-1}$, the inlet temperature was set to 200°C, the column temperature was kept at 50°C for 4 min, then 25 min rises to 220°C, the solvent delay time was 3 min, the transmission line and ion source temperature are set to 230°C and 250°C, respectively (Barros et al. 2013). Electron ionization mass spectra were recorded in Full scan mode. The chemical structure of the degradation product was presumed based on the material retention time and electron mass spectrometry and the NIST database.

Supplementary figures:

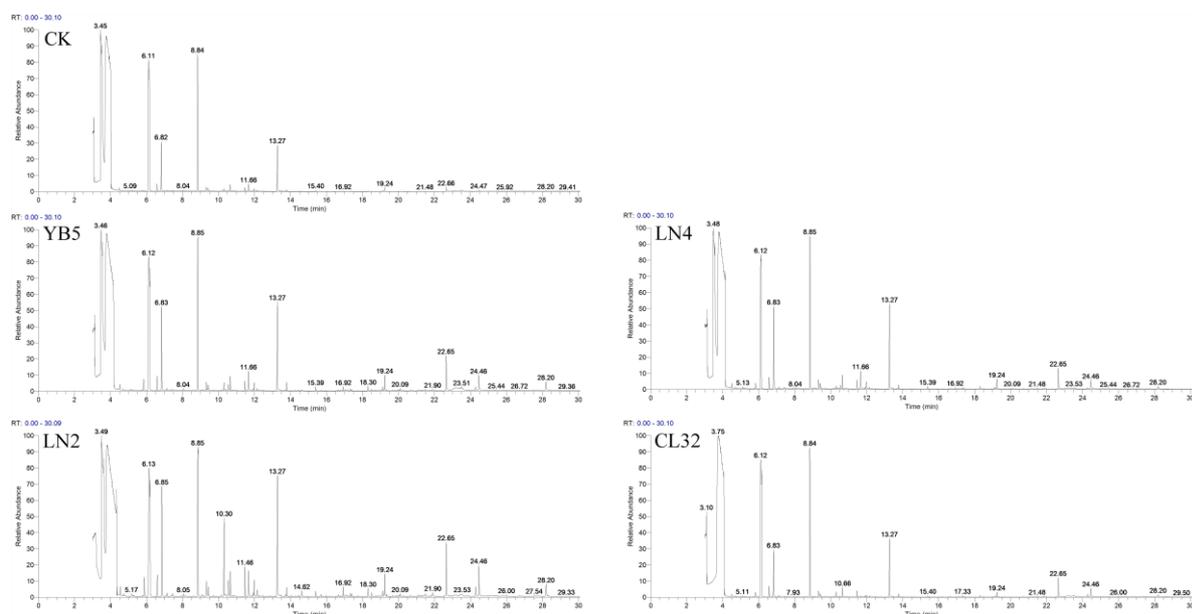


Fig. S1. Chromatograph of compounds extracted with hexane.

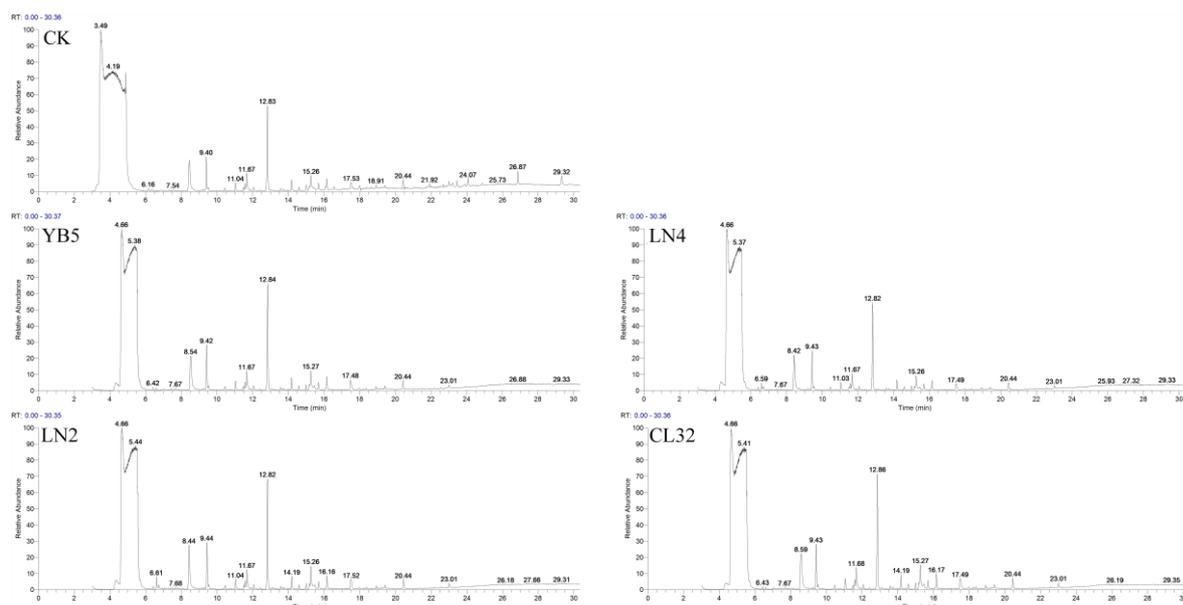


Fig. S2. Chromatograph of compounds extracted with 1,4-dioxane.

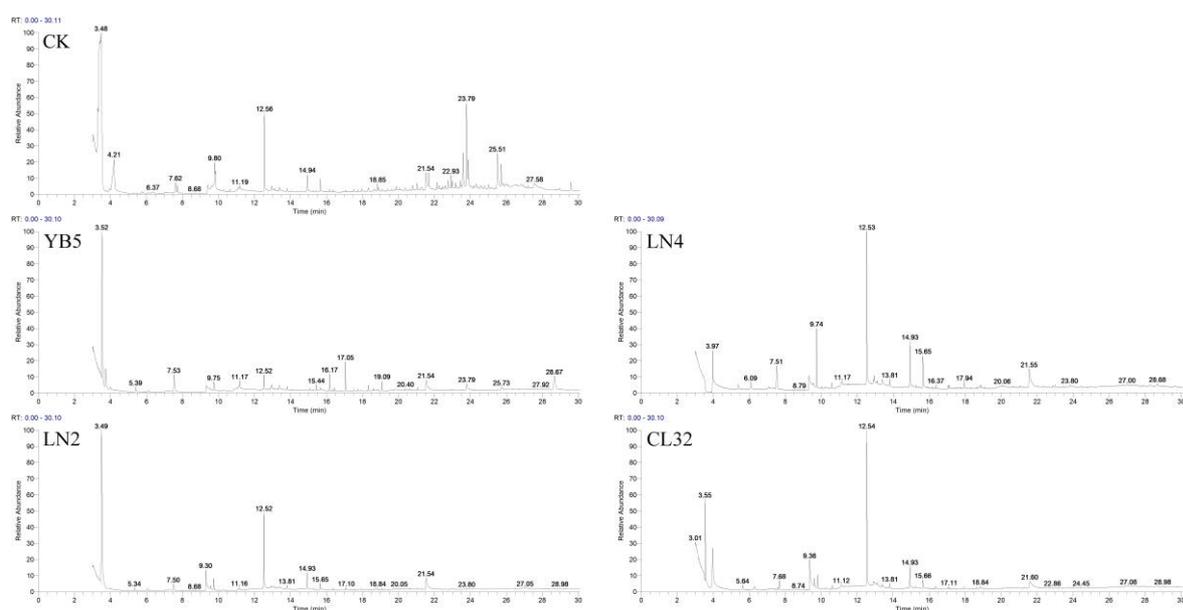


Fig. S3. Chromatograph of compounds extracted with ethyl acetate.

Literature

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