Selective Remove of lignin in Tobacco Stalk by Bacillus and its Laccase Producing Characteristic

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***Abstract* — In order to screen more microorganisms for the biodegradation of tobacco stalk and explore its application value in the reusing of tobacco stalk. One bacterium strain H02 was screened out from soil for detecting its selective degradation of lignin in tobacco stalk and relative higher laccase production when fermentation with tobacco stalk in liquid state. The identification of 16S rDNA sequence indicates that the bacterium is belonged to Bacillus sp. It showed the highest laccase enzyme activity of 332.41 U/mL at 10 d after fermentation and the removal ratio of lignin, cellulose and hemicellulose were 15.68%, 5.58% and 19.66% respectively. In shortly, it can be inferred that this may provided a new approach for the reusing of tobacco stalk.**

Keywords - Bacillus sp., lignin degradation, laccase production, tobacco stalk

I.INTRODUCTION

Lignocellulose is one kind of renewable biological resources and the reuse of lignocellulose in crop stalk has a significant potential for the producing of useful products and bioenergy. According to some statistics, nearly 700 million tons of crop stalk are produced every year in China [1] which make it more meaningful for the reuse research of crop stalk in China.

Due to the exiting of nicotine, solanesol and other chemical materials, tobacco stalk is considered as not only one kind of special biomass resource but also the potential hazards to the environment. In addition, the reusing of tobacco stalk has important implications for the healthy development of agriculture and environment protection. So it is necessary to explore more approaches for the reusing of tobacco stalk. To date, the reusing of tobacco stalk was mainly focused on composting and extracting chemical materials [2, 3] and the pretreatment of tobacco stalk is necessary to break down the lignocellulose structure during the reusing process. Moreover, the biological pretreatment approaches are more environmental, low costing and hydrolysis selected compared with chemical or enzymatic pretreatment [4]. Both fungi and bacterial pretreatment of lignocellulose biomass have been widely studied, but bacteria degradation is considered more advantageous than fungi due to their more adaptation ability to extreme environment and also they can be easily amended for the genetic modification [5].

However, the isolation of bacteria and biodegradation of tobacco stalk by bacteria are not fully studied because of less studies and most of the researches are focused on fungi [6, 7]. Therefore, the purpose of this study was to screen lignin selective degradation bacterium and explore its application value for enzyme production.

II.MATERIAL AND METHODS

A. Soil Samples and Tobacco Stalk

Soil samples were collected from tobacco fields located in Yongzhou city of Hunan province in China. Soil samples were taken at 0-15 cm depth and stored at 4 °C for future use. The tobacco stalk used in this study was taken from Zhucheng city of Shandong province in China and it was milled into pieces by using universal pulverizer (Model LK100) and sieved through 0.28 mm griddle, then it was dried at 105 °C to a constant weight and stored in desiccators for further use.

B. The Screening and Storage of the Strain

The soil samples were dipped into sterile water at a ratio of 1 g/ 10 mL for 8 h and then diluted to 10^{-3}. After that, the diluents were inoculated on the screening medium by spread-plate method. The screening medium was made up by 10 g/L tobacco stalk and 15 g/L agar. The inoculated mediums were cultivated at 37 °C for 48 h and the single colony was picked out onto Luria-Bertani (LB) solid medium (yeast 0.5%, peptone 1%, NaCl 1% and agar 1.5%) for separation and purification. The purified bacterium was cultivated in Luria-Bertani (LB) liquid medium (yeast 0.5%, peptone 1%, NaCl 1%, pH 7.0) for 8-12 h, and then mingled with sterile glycerin (50%) to make the final concentration of glycerin reached to 20-25% and then stored at -80°C.

C. The Identification of the Bacterium

For the identification of the bacteria, the 16S rRNA gene fragment was amplified by polymerase chain reaction (PCR) with a pair of universal primers, fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-CGGCTACCTTGTTACGACTT-3′) as described by Zhang et al [8] and the DNA sequence was determined. We blasted our determined sequence in National Center for Biotechnology Information (NCBI) database and constructed the phylogenetic tree by choosing the highest sequence similarity. The phylogenetic tree was constructed by MEGA 5.10 software selecting Neighbor-joining, 1000 bootstrap and the other parameters were default values.

D. Fermentation of Tobacco Stalk with the Bacterium

The fermentation experiment was carried out in fermentation medium containing 10 g/L tobacco stalk. Furthermore, the bacterium was cultured in LB liquid medium at 37 °C for 12 h for the preparation of inoculation. 100 mL of autoclaved (121 °C, 20 min) fermentation medium (pH 7.0) were inoculated with 1 mL of pregrown pure culture in LB medium using Erlenmeyer flasks (250 mL). The uninoculated fermentation medium with same amount of sterile water was used as a control. The fermentation was carried out at 37 °C on a rotary shaker (180 rpm) in dark condition for 25 days.

E. The Detection of Enzyme Activity

The crude enzyme was collected from fermentation medium every 5 days after inoculation, the fermentation medium was centrifuged at 5000 rpm, 4 °C for 2 min and the supernatant was collected for enzyme activity detection. Lignin peroxidase (LiP, EC1.11.1.14) and manganese peroxidase (MnP, EC1.11.1.13) activity were determined as described by Tien & Kirk et al [9] and Heinfling et al [10]. Laccase (Lac, E.C.1.10.3.2) activity was assayed by monitoring the oxidation of 2′-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) at 420 nm [11]. All spectrophotometric measurements were carried out using a UV-vis spectrophotometer (UV2550 SHIMADZU).

Endoglucanase (CMCase, E.C.3.2.1.4), exoglucanase (C1, E.C.3.2.1.91) and filter paper enzyme activities (FPA) were measured spectrophoto-metrically as described by Beukes and Pletschke [12] based upon the color reaction between the degradation products (glucose) and 3,5-Dinitrosalicylic acid (DNS). In all the cases, one unit of enzyme activity is defined as 1 μmol of glucose equivalents released per min per mL of filtrate under the given conditions. All assays were carried out in triplicate.

F. The Determination of Lignocellulose Content in Tobacco Stalk

The fermentation product was filtered through the filter paper and dried at 60°C to a constant weight. The content of lignin, cellulose and hemicellulose in tobacco stalk were detected through a modified quantitative analysis program described by Van Soest [13]. The relative degradation rate of different components was calculated by comparing with control. All assays were carried out in triplicate.

G. The Determination of Nicotine Content in Tobacco Stalk

The nicotine content in tobacco stalk was detected as described by Wang et al [14]. The nicotine in tobacco stalk was extracted by hydrochloric acid and was measured with a UV-vis spectrophotometer (UV2550) at the given wavelength (236nm, 259nm and 282nm), The content of nicotine in tobacco stalk was calculated by the following formula:

$$\text{Nicotine}(\%) = \frac{1.059 \times [\text{A259} - 0.5 \times (\text{A236} + \text{A282})] \times 100 \times 100}{\text{W} \times (1 - \text{Water}\%) \times 34.3 \times 1000} \quad (1.0)$$

59 is the compensation coefficient; A259, A236 and A282 is the absorbance value of reaction system at 259 nm, 236 nm and 282 nm; W is the weight of sample; 34.3 is the specific extinction coefficient of nicotine.

III. RESULTS AND DISCUSSION

A. The Isolation and Identification of the Bacterium

Until now, a variety of bacteria have been identified as lignocellulose-degrading microorganisms and bacteria belong to *Bacillus*. sp such as *Bacillus licheniformis* [15], *Bacillus halodurans* [16], *Bacillus pumilus* [17]and *Bacillus subtilis* [18] whose are demonstrated to degrade lignocellulose. In our study, one bacterium strain H02 was screened out from soil samples which could use tobacco stalk as a nutrient source of sole carbon and nitrogen. The

determination of its 16S rRNA sequence (NCBI accession number KT025879) revealed that it was belonged to *Bacillus* sp. and the construction of phylogenetic tree indicated that it had the closest genetic relationship with *Bacillus aryabhattai* strain B8W22 (Fig.1). This is the first report about the biodegradation of tobacco stalk by *Bacillus*. sp without supplying external nutrients and it will be provided more theoretical foundation for the degradation of tobacco stalk by bacteria.

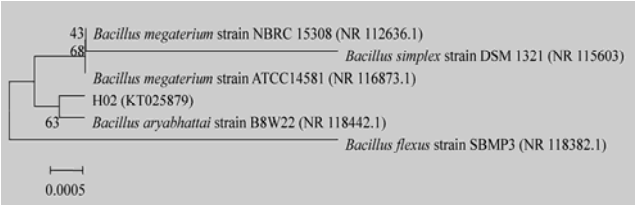

Fig.1 Phylogenetic Tree of *Bacillus* Strain H02 with some Homologous Species. Numbers at Branching Points Refer to Bootstrap Values (1000 resamplings), 0.005 was Sequence Divergence. The Sequences Accession Number in GenBank of the Strains was Shown in Parentheses.

B. Enzyme Activity

Ligninase Enzyme activities: Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) are three kinds of common lignin peroxide enzymes. Initially these enzymes were secreted by fungi [19], but recently bacteria were also reported to produce lignin peroxide enzymes [5]. Our results showed that both Lac and MnP activities were detected in *Bacillus* sp. during the fermentation of tobacco stalk (Fig.2). *Bacillus* sp. have been considered as one kind of high laccase production bacteria and some of them have already been applied for producing of laccase [20], for this reason, we have detected a higher Lac activity than MnP (Fig.2). The large molecular weight of laccase makes it difficult to penetrate into the lignocelluloses, so laccase plays an important role at the initial stage of lignocellulose degradation [5].Our result showed that the highest Lac activity (332.41U/L) was observed at 10 d after inoculation while the highest MnP activity (103.54U/L) was detected at 15 d after inoculation (Fig.2). The unoptimized fermentation conditions may lead to the relatively lower Lac activity compared with similar researches [21].

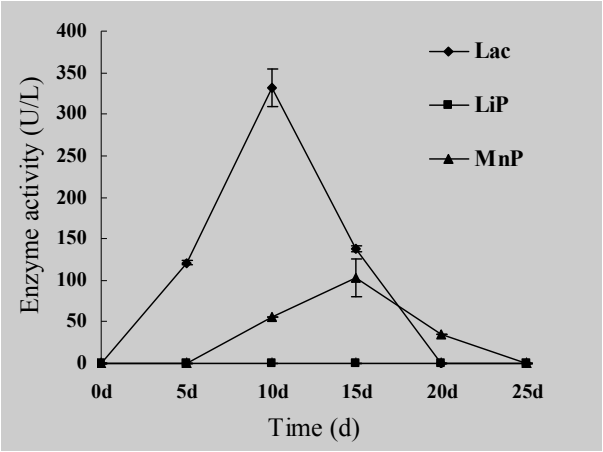

Fig.2 The Lignin Peroxide Enzymes Activities of *Bacillus* Strain H02 during the Liquid Fermentation of Tobacco Stalk

Cellulase activities: It is believed that three kinds of cellulase enzymes were necessary for the degradation of cellulose such as endoglucanase (CMCase, E.C.3.2.1.4), exoglucanase (C1, E.C.3.2.1.91) and β-glucosidase (EC 3.2.1.21). Since the identifying function of β-glucosidase is to cleave cellobiose into glucoses [22], we replaced the β-glucosidase with filter paper enzyme (FPA) which represent the total activity of cellulase. The result indicated that the activities of CMCase, C1 and FPA reached to the highest of 0.78 U/mL, 0.69 U/mL and 0.59 U/mL accordingly 10 days after inoculation (Fig.3). Although this result was in accordance with *Bacillus* sp. SMIA-2 fermentation with sugarcane bagasse [23], it was still at a low level compared with *Bacillus* sp. fermentation with other lignocelluloses biomass such as rice straw or wheat bran. The result of similar cellulase activity with peak level [24] further proved that a 10 day period of inoculation is essential for the cellulase production of *Bacillus*. sp.

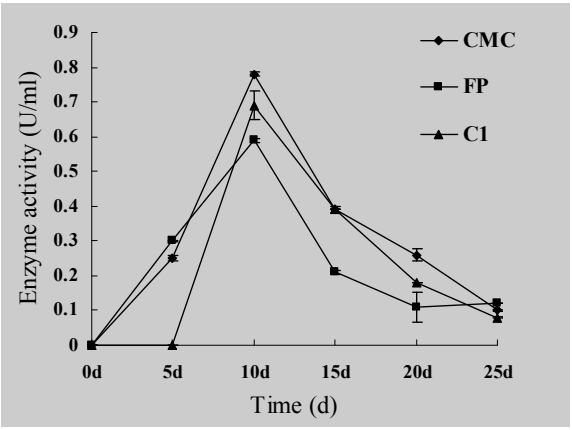

Fig.3 The Cellulase Enzyme Activities of *Bacillus* strain H02 during the Liquid Fermentation of Tobacco Stalk

C. The Degradation of Lignocellulose in Tobacco Stalk

Our experimental tobacco stalk was made up by cellulose (36.2%), hemicellulose (41%), lignin (15.56%), ash and other materials (7.24%). After 25 days fermentation, the content of cellulose, hemicellulose, and lignin in tobacco straw were 34.18%, 32.94% and 13.12% respectively (Fig.4). Besides, the removal ratio of hemicellulose, cellulose and lignin were 19.66%, 5.58% and 15.68% individually. The selective removal of lignin indicated that our screened bacteria could be applied for the delignification of tobacco stalk for the production of biofuels.

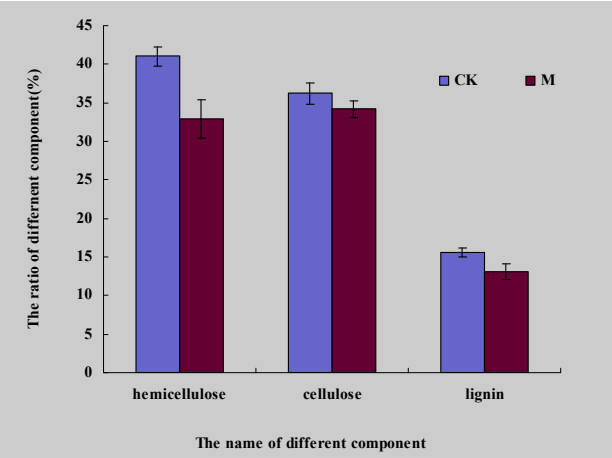

Fig.4 The Content of Hemicellulose, Cellulose and Lignin in Tobacco Stalk 25 d after Fermentation with *Bacillus* Strain H02

D. The Degradation of Nicotine in Tobacco Stalk

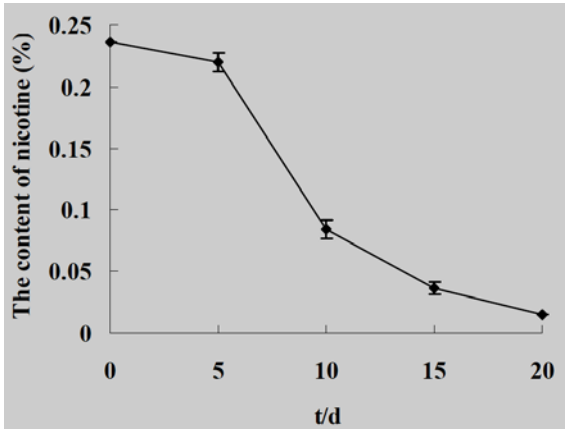

Fig.5 The Content of Nicotine in Tobacco Stalk during the Liquid Fermentation of Tobacco Stalk with *Bacillus* Strain H02

Nicotine is one kind of toxic and hazardous chemical material existing in tobacco stalk which is easily to cause environment pollution. So it is crucial to control the nicotine content during the reusing of tobacco stalk. We found that the *bacillus* strain H02 could degraded the nicotine in tobacco stalk during the fermentation and the content of nicotine in tobacco stalk was lower than 500 mg/kg after 15 days fermentation. Which means the *bacillus* strain could be used for the innocent treatment of tobacco stalk.

IV. CONCLUSION

The identification of screened H02 bacteria strain indicated that it is belonged to *Bacillus* sp. which could grow by using tobacco stalk as a sole carbon and nitrogen source. The fermentation experiment indicated that the bacterium could produce a relatively higher laccase and partially degraded lignin and hemicellulose by fermentation with tobacco stalk. What's more, this bacterium could also degraded nicotine in tobacco stalk. We conceived that this bacterium might be applied to reuse tobacco stalk for laccase production or the selective remove of lignin in tobacco stalk.

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REFERENCE

[1] Y. Lu, N. Li, X. Yuan, B. Hua, J. Wang, M. Ishii, et al., "Enhancing the cellulose-degrading activity of cellulolytic bacteria CTL-6 (Clostridium thermocellum) by co-culture with non-cellulolytic bacteria W2-10 (Geobacillus sp.)," Appl. Biochem. Biotechnol., vol. 171, pp. 1578-1588, Dec 2013.

[2] N. Kopcic, M. Vukovic Domanovac, D. Kucic and F. Briski, "Evaluation of laboratory-scale in-vessel co-composting of tobacco and apple waste," Waste Manag., vol. 34, pp. 323-8, Feb 2014.

[3] A. Piotrowska-Cyplik, A. Olejnik, P. Cyplik, J. Dach and Z. Czarnecki, "The kinetics of nicotine degradation, enzyme activities and genotoxic potential in the characterization of tobacco waste composting," Bioresour. Technol., vol. 100, pp. 5037-5044, Nov 2009.

[4] M. Taha, E. Shahsavari, K. Al-Hothaly, A. Mouradov, A. T. Smith, A. S. Ball, et al., "Enhanced biological straw saccharification through coculturing of lignocellulose-degrading microorganisms," Appl. Biochem. Biotechnol., vol. 175, pp. 3709-3728, 2015.

[5] J. Li, H. Yuan and J. Yang, "Bacteria and lignin degradation," Frontiers of Biology in China, vol. 4, pp. 29-38, 2008.

[6] N. Zhang, D. Liu, X. Yang, Y. Xu, Q. Shen and Q. Huang, "Screening of thermophilic cellulose-decomposing fungi and their efficiency in decomposing tobacco stems," Acta Scientiae Circumstantiae, vol. 30, pp. 549-555, 2010.

[7] X. Yang, Y. Liu, Z. Liu, Y. Lai and Y. Chen, "High yield laccase producing strains screened and the study of tobacco stem degradation affected by the strains," Chinese Agricultural Science Bulletin, vol. 30, pp. 52-57, 2014.

[8] H. Zhang, M. Cao, X. Jiang, H. Zou, C. Wang, X. Xu, et al., "De-novo synthesis of 2-phenylethanol by Enterobacter sp. CGMCC

5087," BMC biotechnology, vol. 14, pp. 30, 2014.

[9] M. Tien and T. K. Kirk, "Lignin-degrading enzyme from Phanerochaete chrysosporium: Purification, characterization, and catalytic properties of a unique H(2)O(2)-requiring oxygenase," Proc Natl Acad Sci USA, vol. 81, pp. 2280-2284, Apr 1984.

[10] A. Heinfling, M. J. Martinez, A. T. Martinez, M. Bergbauer and U. Szewzyk, "Transformation of industrial dyes by manganese peroxidases from Bjerkandera adusta and Pleurotus eryngii in a manganese-independent reaction," Appl Environ Microb, vol. 64, pp. 2788-2793, Aug 1998.

[11] A. P. Michael, R. Rosa, T. Raunel and V.-D. Rafael, "Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by Coriolopsis gallica UAMH 8260 laccase," Appl Environ Microb, vol. 65, pp. 3805-3809, 1999.

[12] N. Beukes and B. I. Pletschke, "Effect of sulfur-containing compounds on Bacillus cellulosome-associated 'CMCase' and 'Avicelase' activities," Fems Microbiol Lett, vol. 264, pp. 226-231, Nov 2006.

[13] P. J. Van Soest, J. B. Robertson and B. A. Lewis, "Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition," Journal of Dairy Science, vol. pp. 3583-3597, 1991.

[14] R. Wang, F. Han and S. Yang, "Tobacco chemical quality analysis methods," Zhengzhou, Henan Science and Technology Press, 1999,

[15] K. Bala-Amutha and A. G. Murugesan, "Biohydrogen production using corn stalk employing Bacillus licheniformis MSU AGM 2 strain," Renewable Energy, vol. 50, pp. 621-627, 2013.

[16] X.-q. Lin, S.-y. Han, N. Zhang, H. Hu, S.-p. Zheng, Y.-r. Ye, et al., "Bleach boosting effect of xylanase A from Bacillus halodurans C-125 in ECF bleaching of wheat straw pulp," Enzyme Microb Tech, vol. 52, pp. 91-98, 2013.

[17] J. Lü and P. Zhou, "Optimization of microwave-assisted $FeCl_3$ pretreatment conditions of rice straw and utilization of Trichoderma viride and Bacillus pumilus for production of reducing sugars," Bioresour. Technol., vol. 102, pp. 6966-6971, 2011.

[18] S. Yadav and R. Chandra, "Syntrophic co-culture of Bacillus subtilis and Klebsiella pneumonia for degradation of kraft lignin discharged from rayon grade pulp industry," Journal of Environmental Sciences, vol. 33, pp. 229-238, 2015.

[19] A. J. Chang, J. Fan and X. Wen, "Screening of fungi capable of highly selective degradation of lignin in rice straw," Int. Biodeterior. Biodegrad., vol. 72, pp. 26-30, 2012.

[20] N. Loncar, N. Bozic, J. Lopez-Santin and Z. Vujcic, "Bacillus amyloliquefaciens laccase--from soil bacteria to recombinant enzyme for wastewater decolorization," Bioresour. Technol., vol. 147, pp. 177-183, Nov 2013.

[21] M. Kuddus, B. Joseph and P. Wasudev Ramteke, "Production of laccase from newly isolated Pseudomonas putida and its application in bioremediation of synthetic dyes and industrial effluents," Biocatalysis and Agricultural Biotechnology, vol. 2, pp. 333-338, 2013.

[22] A. Karim, M. A. Nawaz, A. Aman and S. A. Ul Qader, "Hyper production of cellulose degrading endo (1,4) β-d-glucanase from Bacillus licheniformis KIBGE-IB2," Journal of Radiation Research and Applied Sciences, vol. 8, pp. 160-165, 2015.

[23] S. A. Ladeira, E. Cruz, A. B. Delatorre, J. B. Barbosa and M. L. L. Martins, "Cellulase production by thermophilic Bacillus sp. SMIA-2 and its detergent compatibility," Electronic Journal of Biotechnology, vol. 18, pp. 110-115, 2015.

[24] S. Sadhu, P. K. Ghosh, T. K. De and T. K. Maiti, "Optimization of cultural condition and synergistic effect of lactose with carboxymethyl cellulose on cellulase production by Bacillus sp. isolated from fecal matter of elephant (Elephas maximus)," Advances in Microbiology, vol. 03, pp. 280-288, 2013.