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Original paper

Detoxification of corn stalk pretreated with calcium hydroxide for true protein accumulation

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Abstract Alkaline pretreatment is essential in lignin degradation, but the inhibitors produced in this process affect microbial growth. To overcome the impacts of the phenolic compounds, detoxification was applied to corn stalk pretreated with calcium hydroxide.The results showed that ferulic acid degradation rate can reach 85.11% by laccase at the optimal conditions. *Phanerochaete chrysosporium* degraded most vanillin (77.19%) and p-hydroxybenzaldehyde (63.82%) but the degradation of ferulic acid (15.34%) was relatively weak. Laccase combined with *Phanerochaete chrysosporium* detoxified most of the phenolic compounds including 2-methoxy-4-vinylphenol (88.46%) and salicylic acid (58.13%) that hardly decompose alone after calcium hydroxide pretreatment in this study. These results inferred that *Phanerochaete chrysosporium* might generate some substance during the spore germination and growth period which may cooperate with laccase to decompose the phenolic compounds. After the fermentation of detoxified corn stalk by *Neurospora crassa*, the true protein content was increased by 2.73 times, and 21.17% lignin was degraded.

Keywords alkaline pretreatment, inhibitor, detoxification, laccase, *Phanerochaete chrysosporium*

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Introduction

Corn stalks are widely used for ruminant animal feedstuffs owing to the serious shortage of coarse fodder in China, and approximately 230 million tons of corn stalk were produced in China every year (Cai, 2016). With the development of agricultural technology and the expansion of crop cultivation, more and more agricultural waste is produced (Gómez-Muñoz, 2017). The unreasonable use of agricultural wastes has caused great pollution to the environment. The conversion of agricultural wastes into animal feedstuffs is a good way to promote the operation of agroecosystems, which is also an effective approach for environment protection. However, there are some problems associated with original corn stalks, such as low digestibility coefficient, poor palatability, and shortage of true protein, vitamin, calcium, magnesium, copper or zinc. Moreover, currently used silage or micro-storage corn stalk also need to be improved in the aspects of digestibility coefficient, lignin degradation rate and nutrient value, especially true protein (Jia, 2017). Therefore, the technology for corn stalks processing to produce digestible, high nutritional value animal feedstuffs is very important for rational utilization of agricultural wastes.

The existence of lignin prevents ruminants from digesting corn stalks well (Jia, 2017). Thus, bioconversion of lignocellulosic material into valuable products requires the degradation of lignin by pretreatment to make the polysaccharides more accessible to the hydrolysis steps (Ventorino, 2016). Pretreatment of lignocellulosic materials is therefore essential for efficient enzymatic hydrolysis (Yang, 2017; Venturin, 2018). In previous literatures, various pretreatment techniques on lignocellulosic materials have been studied (Puligundla, 2016; Raud, 2016; Rouches, 2016; Guerrero, 2017; Karapatsia, 2017; Hu, 2018; Qiao, 2018; Wu, 2019; Li, 2019).Alkaline pretreatment have advantages compared with acid pretreatment, i.e., alkaline pretreatments have less sugar degradation, which avoids the formation of furan derivative and can recover many of the caustic salt (Jin, 2013). The most significant impact of alkaline pretreatment is to effectively break down lignin seal, disrupt crystalline structure of cellulose, and make pretreated fibers more accessible to enzymatic digestion (Sun, 2014; Yu, 2014; Jin, 2016; Romero-Guiza, 2017; Hao, 2019). Alkaline pretreatment agents include sodium hydroxide, potassium hydroxide, calcium hydroxide, and ammonium hydroxide, sodium hydroxide pretreatment is one of the most frequently studied methods in recently years (Talebnia, 2010). However, calcium hydroxide is also an effective pretreatment agent and is the least expensive per kilogram of hydroxide (Parveen Kumar, 2009). However, alkaline pretreatment generates inhibitory compounds, which may inhibit microbial growth (Hendriks, 2009; Liu, 2019), leading to low yield of true protein. Detoxification is required to eliminate the inhibitory effects of the toxics. Generally, the pretreated materials were washed for detoxification (Toquero, 2014). However, this procedure not only requires abstersion, filtration and compression in the process, but also consumes large quantities of water and causes pollution(Yang, 2011). The process of pretreatment by calcium hydroxide can avoid washing, as calcium residues in corn stalk can be utilized by ruminants. Free or immobilized laccase has been used to remove the toxics, but it can only selectively remove part of the toxics (Ludwig, 2013). *Phanerochaete chrysosporium* was rarely used to remove toxics from alkaline hydrolysates of corn stalks. There was almost little report about pretreatment both with laccase and *P. chrysosporium* to remove the toxics in the alkaline hydrolysates.

In this study, the changes of lignocellulosic content before and after $Ca(OH)_2$ pretreatment was assayed. The contenl of phenolic compounds in pretreatment hydrolysate was also measured. Detoxification was carried out by laccase and aureospora, and the degradation of lignin and the accumulation of true protein were evaluated after fermentation.

Materials and Methods

Preparation of samples

The corn stalks were smashed, washed and dried at 45℃, and then minced into particles < 5 mm in size and were kept in dry place at room temperaturet. All chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd of China and were of analytical grade.

Corn stalk pretreatment

Chemical pretreatment is one of the methods with the greatest application potential to improve the biodegradability of cellulose by removing lignin and/or hemicelluloses (Behera, 2014). Thus, alkaline pretreatment with $Ca(OH)_2$ have been used in this study. 5 g corn stalk was added into 100 mL distilled water with 0.5 g $Ca(OH)_2$ and mixed thoroughly and the solid part was used to analyze the chemical composition after incubation in a water bath at 20℃, 40℃, 60℃, 80℃, 100℃ and 120℃, respectively. The corn stalk were pretreated by $Ca(OH)_2$ (0.25%, 0.5%, 0.75%, 1.0% (w/v)) under 5% (w/v) solid loading which placed in 250 mL Erlenmeyer flasks in a solid: liquid ratio of 1:20 (w/w), and the solid-liquid mixture was hydrolyzed in water bath (80℃) for 1 h. After pretreatment, solid-liquid separation was performed by 200 mesh strainers. The liquid part was used to extract the phenolic compounds by dichloromethane and the phenolic compounds were detected by High Performance Liquid Chromatography (HPLC).

Detoxification

Although alkaline pretreatment could remove lignin and hemicelluloses, improve the biomass digestibility, some compounds such as phenolic compounds can be generated during this process (Zhang, 2014). To avoid the inhibition of microbial growth of by toxic compounds, detoxification was used to reduce the phenolic compounds. And detoxification was divided into two steps, the first step was carried out at 30℃, 200 rpm for 1 h and the response surface methodology (RSM) was used to optimize the ferulic acid degradation rate. The solid proportion was 5%

(w/v) and the activity of laccase was 2000 U/g of solid. And we conducted a Box-Behnken design (BBD) to determine the optimal ferulic acid degradation rate of the pH, Copper ion and Manganese ion concentration. Second step was carried out at 30℃, 150 rpm for 24 h with *P. chrysosporium* (0.5 mL of spore suspension in 100 mL hydrolysate). Two steps detoxification were compared with laccase or *P. chrysosporium* alone to compare the effect of the detoxification. Triplicate experiments were performed. After detoxification, the liquid fraction was collected for composition analysis and the solid part was collected and dried in oven at 45℃ after detoxification.

P. chrysosporium, N. crassa used in this study were laboratory stored and the laccase was purchased from Xiasheng Industrial Co., Ltd, Ningxia, China. *P. chrysosporium, N. crassa* were grown on potato dextrose agar (PDA) plates and incubated at 30℃ for 3-5 d. Then, the spores were collected with distilled water to spore suspension. The solid-state fermentation substrate was made up by detoxification corn stalk (5 g), urea (0.5%), ammonium sulfate (2%), glucose (2%) and water added to a 67% moisture content. The fermentation substrate was sterilized by autoclaving at 120℃ for 30 min, and then cooled prior to inoculation. The sterilized fermentation substrate was inoculated with *N. crassa* spore suspension (0.1mL of spore suspension per 1 g dry corn stalk), and the substrate was incubated at 30℃ for 3 days.

Analytical methods

Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL) and Acid Insoluble Ash (AIA) were determined in corn stalk by ANKOM Technology Methods (McIntosh, 2011). Phenolic compounds in pretreatment hydrolysate were measured through high performance liquid chromatography (HPLC) using a Bischoff ProntoSIL C18 column, an ultraviolet-visible detector (UVD) and 1% acetic acid as mobile phase A, methanol as mobile phase B at a flow ratio of 1.0 mL/min and room temperature. Dichloromethane was used to extract the phenolic compounds in the samples (Iqbal, 2013). Response surface methodology (RSM) is a suitable statistical approach to judge the importance of each individual parameter and their interactions on response variables (Kim, 2012). To shorten the detoxification time, three factors were used: pH (X_1), Copper ion (X_2), Manganese ion (X_3). Test condition varied: pH 3-5, Copper ion concentration of 20-40 mg/L, Manganese ion concentration of 20-40 mg/L. A total of 17 experiments run with varied values of the three variables were actualized by BBD. And Design-Expert Software (Version 8.0.6) was used to accomplished the data analysis. True protein content were obtained by using Automatic Kjeldahl Apparatus (Lonnerdal, 2017). All analyses were performed in triplicate.

Results and discussion

Chemical composition of corn stalks

The composition of crushed corn stalk used in this study. Neutral Detergent Fiber (NDF) is the residues after digesting in a neutral detergent solution, and the fiber residues are predominantly hemicellulose, cellulose and lignin. The Acid Detergent Fiber (ADF) is the residues after digesting with 0.5 mol/L H_2SO_4 and 2% CTAB, and the fiber residues are predominantly cellulose and lignin. Acid Detergent Lignin (ADL) is determined gravimetrically as the residues upon ignition after 72% H_2SO_4 treatment. The hemicellulose is the major component at 44.27% and the cellulose is 33.54%, acid insoluble lignin levels is 7.70%.

Effects of different temperature pretreatment on corn stalk

Alkaline pretreatment has advantages compared with acid pretreatment and liquid-hot-water pretreatment (Park, 2010; Cai, 2016). However, severe alkaline pretreatment could cause the serious loss of carbohydrates (Sierra, 2009). Thus, mild alkaline pretreatment with $Ca(OH)_2$ have been used in this study. A 5 g corn stalk was added into 100 mL distilled water with 0.5 g $Ca(OH)_2$ and mix thoroughly. After incubation in a water bath at different temperature for 1 h, the solid part was used to analysis the chemical composition. The temperature of P1-P6 was 20℃, 40℃, 60℃, 80℃, 100℃, 120℃ and N was the untreated corn stalk (Fig.1). According to the reduction of the lignocellulose and the cost factor, the greatest degree of lower cost of power and enhance the degradation of lignin and hemicellulose, we choose the 80℃ as the optimal pretreatment temperature. The hemicellulose and lignin degradation rate could reach 39.00%, 21.17% respectively.

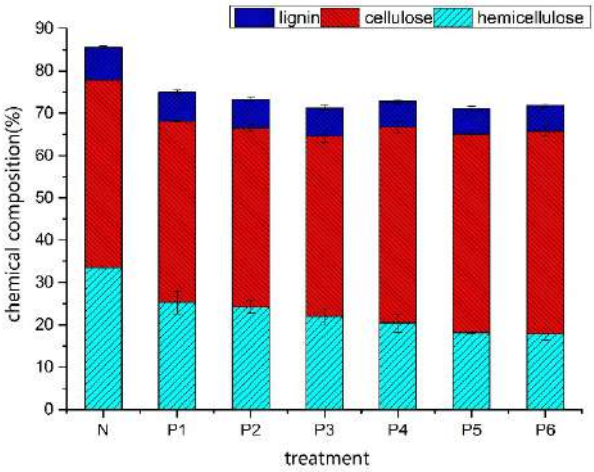

Figure 1 The chemical composition of corn stalk after different temperature pretreatment compared to untreated samples.

Effects of different Ca(OH)2 concentration pretreatment on corn stalk

Corn stalk was pretreated by $Ca(OH)_2$ (0 g/L, 2.5 g/L, 5 g/L, 7.5 g/L, 10 g/L) under 10% (w/v) solid proportion for an hour in a water bath at 80℃. After pretreatment, solid-liquid separation was performed by 200 mesh strainers. The liquid part was used to extract the phenolic compounds by dichloromethane and the phenolic compounds were detected by HPLC. Lignin was the main source of phenolic compounds (Jonsson, 2013; Yang, 2017), so the content of the phenolic compounds could represent the degree of

degradation of lignin. Five main phenolic compounds (vanillin, p-hydroxybenzaldehyde, ferulic acid, 2-methoxy-4-vinylphenol, salicylic acid) were detected and the standard curve was adopted to determinate the contents. According to the inhibitors in hydrolysates, we choose the 5 g/L $Ca(OH)_2$ as the optimal concentration, as it is shown in Table 1. The phenolic compounds could reach to a maximum quantity when the concentration of $Ca(OH)_2$ was 5 g/L.

Table 1. Inhibitors concentration in corn stalks hydrolysates subjected to different $Ca(OH)_2$ concentration

$Ca(OH)_2$ Concentration(g/L)	**Inhibitors in hydrolysates(μg/mL)**				
	Vanillin	P-hydroxybenzaldehyde	Ferulic acid	2-methoxy-4-vinylphenol	Salicylic acid
0	3.86±0.68	1.71±0.42	11.58±0.82	3.11±0.85	1.03±0.57.
2.5	12.38±1.05	12.43±0.66	75.45±1.34	9.30±1.56	13.77±3.54
5.0	14.60±1.26	14.87±1.34	111.17±3.42	5.20±1.39	12.80±2.96
7.5	15.22±0.83	13.30±1.06	110.89±2.86	1.89±0.94	9.17±2.42
10.0	13.66±0.78	12.60±0.95	98.03±2.35	2.78±0.82	5.16±1.85

Table 2. Projects and results of response surface experiments

Run	X1: pH	X2: Copper ion	X3: Manganese ion	Ferulic acid degradation rate (%)
1	3.0	20	30	46.12
2	5.0	40	30	41.42
3	3.0	40	30	36.69
4	4.0	20	20	31.26
5	4.0	30	30	84.39
6	4.0	40	40	27.96
7	3.0	30	20	44.00
8	5.0	30	20	28.49
9	3.0	30	40	47.62
10	5.0	20	30	35.75
11	4.0	30	30	84.39
12	4.0	30	30	84.39
13	4.0	30	30	84.39
14	5.0	30	40	41.47
15	4.0	20	40	46.95
16	4.0	30	30	84.39
17	4.0	40	20	29.30

Table 3. ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Mean Square	F Value	p-value Prob>F
Model	8115.04	901.67	90.62	<0.0001
X_1-pH	93.16	93.16	9.36	0.0183
X_2-Copper ions	76.32	76.32	7.67	0.0277
X_3-Manganese ions	119.74	119.74	12.03	0.0104
X_1X_2	57.00	57.00	5.73	0.0479
X_1X_3	21.90	21.90	2.20	0.1815
X_2X_3	72.51	72.51	7.29	0.0307
X_1^2	1509.42	1509.42	151.70	<0.0001
X_2^2	2729.58	2729.58	274.33	<0.0001
X_3^2	2644.49	2644.49	265.78	<0.0001
Residual	69.65	9.95		
Lack of Fit	69.65	23.22		
Pure Error	0.000	0.000		
Cor Total	8184.69			

Optimization of the laccase detoxification condition using RSM

According to the experimental scheme given by BBD design, the corresponding experiments were carried out. Table 2 lists the various combinations of three independent variables and their experimental data. The maximum ferulic acid degradation rate reached to 84.39%. And the predicted ferulic acid degradation rate was calculated with the following quadratic equation:

$$Y=84.39-3.41X_1-3.09X_2+3.87X_3+3.77X_1X_2+2.34X_1X_3-4.26X_2X_3-18.93X_1^2-25.46X_2^2-25.06X_3^2$$

Where Y is the ferulic acid degradation rate, X_1 is pH, X_2 is copper ion concentration, mg/L, and X_3 is manganese ion concentration, mg/L. Significance in our quadratic model could be demonstrated by desirable F-value, P-value, the lack of fit, and the adjusted determination coefficient (Adj R^2). X_1, X_2, X_3, X_1X_2, X_2X_3, X_1^2, X_2^2, X_3^2 were proven to be the significant terms with the p values below 0.05 (Table 3). The optimal pretreatment condition for maximum ferulic acid degradation rate was X_1=3.91, X_2=29.3 mg/L, X_3=30.8 mg/L, which would result in a predicted ferulic acid degradation rate to 84.82%. To test and verify the result of the predicted value, the experiments were carried out at the optimal conditions, showing the ferulic acid degradation rate reach to 85.11% ±1.08%.

The outcomes of the experiment revealed three dimensional response surface plots indicating the correlation between two variable quantities with one variable being kept immobile in its optimal condition (Kim, 2012). There is a vertex of each response surface plot, indicating that maximum ferulic acid degradation rate could be obtained inside the design boundary. The interactions of pH, copper ion concentration, manganese ion concentration for ferulic acid degradation rate were represented in Fig.2.

Under these selected conditions, the ferulic acid was 85.11% degradation by laccase in 1 h. This resulted in greatly reducing the time of laccase detoxification.

Detoxification methods on phenolic compounds

In recent years multifarious methods have been used to conquer the inhibition effect of fungal growth (Rasmussen, 2017). In this study, ferulic acid degradation rate could reach to 85.11% by laccase at the optimal conditions (Cu^{2+}:29.3 mg/L, Mn^{2+}:30.8 mg/L, pH 3.9, 30℃,1 h), but at the same time, an unknown substance which might be a phenolic was produced in this process and degradation to other phenolic compounds was found to be inconspicuous. *P. chrysosporium* could degrade the most vanillin (77.19%) and p-hydroxybenzaldehyde (63.82%) but the degradation of ferulic acid (15.34%) was relatively weak. According to the phenolic compounds degradation rate, laccase combined with *P. chrysosporium* could decomposed the maximal part of the toxic compounds, and more effectively than used singly. The degradation rates of vanillin, p-hydroxybenzaldehyde, ferulic acid, 2-methoxy-4-vinylphenol and salicylic acid could reach 89.66%, 90.99%, 98.00%, 88.46%, 58.13% respectively (Fig.3). And the unknown substance was also being degraded. During the detoxification of *P. chrysosporium*, no lignin peroxidase or manganese peroxidase activity were detected. This mean that *P. chrysosporium* might generate some substance which could cooperate with laccase to decompose the phenolic compounds during germination and growth period. It's interesting that 2-methoxy-4-vinylphenol and salicylic acid can only be degraded by combined action. And the degradation rate of vanillin, p-hydroxybenzaldehyde and ferulic acid were further improved.

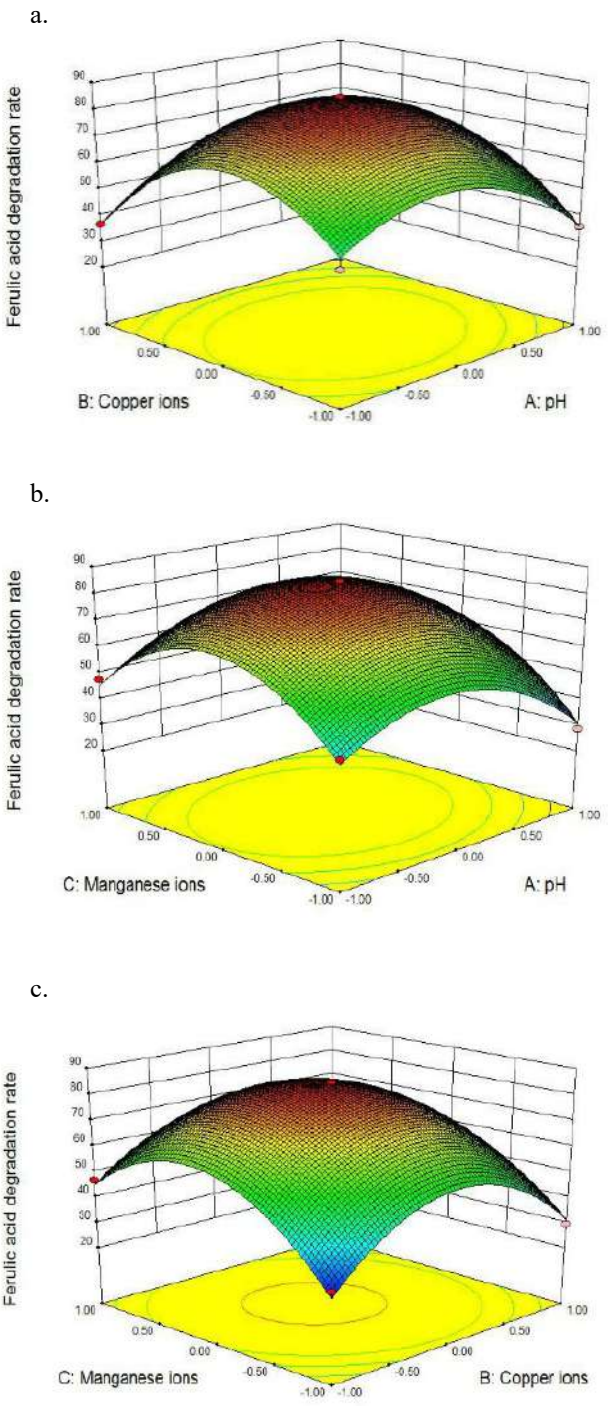

Figure 2. Response surface of $Y=f(X_1,X_2)$(a, X_3=30mg/L), $Y=f(X_1,X_3)$(b, X_2=30mg/L), $Y=f(X_2,X_3)$(c, X_1=4). X_1, pH, X_2, copper ion concentration, X_3, manganese ion concentration

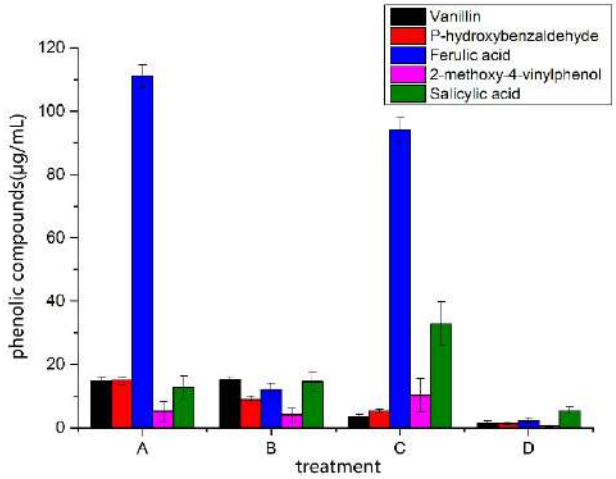

Figure 3. Phenolic compounds concentration in hydrolysate after different detoxification methods. A: no detoxification; B: detoxification with laccase; C: detoxification with *P. chrysosporium;* D: detoxification with laccase and *P. chrysosporium.*

True protein production by Neurospora crassa fermentation

The solid part was collected to oven dry at 45°C after detoxification. The solid-state fermentation substrate was made up by detoxification corn stalk (5 g), urea (0.5%), Ammonium sulfate (2%), glucose (2%) and water added to a 67% moisture content. The fermentation substrate was sterilized by autoclaving at 120°C for 30 min, and then cooled prior to inoculation. The sterilized fermentation substrate was inoculated with *N. crassa* spore suspension (0.1 mL of spore suspension per 1 g dry corn stalk), the substrate was incubated at 30°C for 3 days. The same levels without detoxification corn stalk as control. It was shown that the *N. crassa* growth condition on the detoxification corn stalk is much better than the non-detoxification one, the true protein content increased by 2.73 times.

Conclusion

Laccase and *P. chrysosporium* had detoxify the most of the phenolic compounds that can't be detoxified alone after calcium hydroxide pretreatment in this study. The results inferred that *P. chrysosporium* might generate some substance which could cooperate with laccase to decompose the phenolic compounds during its spore germination and growth period (but no lignin peroxidase or manganese peroxidase).The results indicated that two steps detoxification methods with laccase and *P. chrysosporium* could improve the fermentation efficiency of corn stalk pretreated with calcium hydroxide, which could be used to produce the digestible, high nutritional value animal feedstuffs.

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