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Efficiency and mechanisms**

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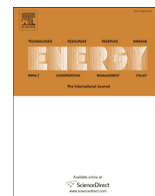
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White rot fungi pretreatment to advance volatile fatty acid production from solid-state fermentation of solid digestate: Efficiency and mechanisms

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ABSTRACT

Anaerobic digestion has been widely applied throughout the world for lignocellulosic biomass treatment and energy recovery. However, the solid digestate from anaerobic digestion still contains a rather large fraction of poorly anaerobic degradable lignocellulosic fibers due to inhibition of lignin, which deeply limits the bioenergy production from lignocellulosic biomass. Therefore, a novel fungal pretreatment method using *P. sajor-caju* and *T. versicolor* was investigated to advance the solid-state fermentation of solid digestate and improve the production of fermentative volatile fatty acids (VFAs). The results showed that a maximum VFA yield of 240 mg COD/g VS was obtained from solid digestate pretreated by *P. sajor-caju* in 6 weeks, which was 1.17-fold and 1.24-fold higher than that of the autoclaved group and raw substrate, respectively. The mechanisms indicated that these fungal strains could grow on the solid digestate and secrete ligninolytic enzymes such as laccase and manganese peroxidase to degrade lignin in different extents. Besides, fungal pretreatment substantially changed the solid digestate characteristics such as cellulose/lignin ratio and the presence of specific functional groups. Moreover, fungal pretreatment using *P. sajor-caju* effectively damaged the structure and increased surface area and pore size of the solid digestate, which is beneficial to further VFA production.

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1. Introduction

As an efficient and relatively simple biotechnology, anaerobic digestion has been widely applied throughout the world for the stabilization of, and energy recovery from lignocellulosic wastes [1,2]. However, the conversion efficiency of anaerobic digestion process largely depends on the presence and intertwining of recalcitrant fibrous matters in the substrate biomass [3]. After digestion, the solid digestate that is separated from the liquid fraction by dewatering contains a rather large fraction of the poorly anaerobic degradable lignocellulosic fibers. The observed

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recalcitrance of these polymers is due to their complex, poly-morphic structure and high degree of aromaticity of the lignin part. Therefore, solid digestate is usually considered to be unsuited for further conversion into more value-added products and generally finds its way to application in the agricultural sector for soil conditioning or animal bedding, or the solid digestate is incinerated [4].

However, with suitable pretreatment the residue still could be further utilized and converted into higher value-added products. Various pretreatment methods such as physical, chemical and biological ones have been widely studied [5–7]. One biological method uses white rot fungi, which is considered a potentially economical and environment friendly approach compared with traditional physical and chemical methods, due to its low consumption of energy and chemicals. Owing to their complex non-specific extracellular enzymes, white rot fungi have the ability to change their chemical composition and degrade lignocellulosic biomass [8].

Recently, production of volatile fatty acids (VFAs) instead of

biogas from organic residues as a product during anaerobic digestion has been regarded promising [9]. VFAs are considered bulk chemicals to produce high-value-added products such as polyhydroxyalkanoates (PHA), a precursor for bioplastics, etc. Moreover, harvested VFAs from sludge digestate in wastewater treatment plants (WWTPs) could also be used as carbon source in enhanced nutrient removal processes, which would enhance the sustainability of WWTPs [10]. Besides, anaerobic fermentation of organic residues to produce VFAs offers the advantage that almost all the chemically enclosed energy of the substrate is conserved in the products. In comparison to biogas production, VFA production can be realized at much shorter retention time, requiring smaller reactor unit.

Although a few studies have been carried out to produce ethanol or methane from the solid fraction of digestate produced from various substrates, such as manure [11,12], research and knowledge on VFA production specifically with fungal pretreatment from these solid wastes is very limited. Hence, the aim of this study was to investigate the role of two different white rot fungi in improving the anaerobic degradability of solid digestate, and enhancing the fermentative VFA production. Furthermore, the mechanisms involved in the fungal pretreatment were deciphered.

2. Materials and methods

2.1. Fungal strains and spawn

Two white rot fungi, *Trametes Versicolor* (*T. versicolor*, strain MES 11914) and *Pleurotus Sajor Caju* (*P. sajor-caju*, strain MES 03464), were employed in this study. The fungi were initially cultured and incubated on 3% malt extract agar medium (pH \approx 5.0) and at 25 °C until mycelia covered most of surface of plates. Then spawn was prepared by adding five pieces of colonized agar (1.5 \times 2.0 cm) into sterilized sorghum grains and incubating at 25 °C until grains were colonized by the mycelia. Afterwards, the spawn was stored in a fridge at 4 °C for further use.

2.2. Solid digestate and inoculum

Solid digestate used in the present study as substrate was collected from a co-digestion biogas plant located in Waalwijk, the Netherlands, which mainly treats agricultural residues such as wasted fruits and vegetables residues. The substrate was initially dried at 60 °C for 24 h, and subsequently chopped into small pieces with a size between 2 and 3 cm. Inoculum used in this study was collected from an anaerobic digester of a local WWTP (Harnaspolder, Den Hoorn, the Netherlands) that treats primary and secondary sludge. Table 1 summarizes the basic characteristics of the solid digestate and the inoculum.

2.3. Fungal pretreatment

Around 100 g (dry matter) of substrate was weighed into autoclavable polypropylene containers (1.2 L) with a filter cover (Combiness, Nazareth, Belgium). The substrate and containers were sterilized by autoclaving for 30 min at 121 °C. After the autoclaved containers were cooled to room temperature, the spawn was added to the sterilized substrate at a weight ratio of 0.1 (0.1 g wet weight of spawn per g dry matter of substrate) and mixed under sterile conditions. Then, the mixed samples were incubated at 25 °C and at a relative humidity of 70% in a climate-controlled chamber (HPP110, Memmer Company, Germany). Samples for analysis were taken under sterile condition every week during pretreatment.

Table 1
Basic characteristics of solid digestate and inoculum.

Components	Solid digestate	Inoculum
Total solid (TS) ^a g/kg	296.3 \pm 0.2	27.3 \pm 0.1
Volatile solid (VS) ^a g/kg	257.1 \pm 0.2	19.2 \pm 0.1
VS/TS (%)	86.80 \pm 0.01	70.01 \pm 0.10
pH	ND	7.85 \pm 0.03
Total carbon ^b (%)	44.1 \pm 0.5	ND
Cellulose ^b (%)	18.1 \pm 0.3	ND
Hemicellulose ^b (%)	14.6 \pm 0.5	ND
Lignin ^b (%)	31.1 \pm 0.4	ND
Total nitrogen ^b (%)	2.4 \pm 0.3	ND
C/N	18.2 \pm 0.3	ND
Total phosphorus ^b (%)	0.95 \pm 0.02	ND

ND represents not determined. The data are shown as average \pm standard deviations of three replicates.

^a Represents the measurements based on wet weight.

^b Represents the measurements based on dry weight.

2.4. Batch assays of anaerobic fermentation

Anaerobic fermentation was operated with a TS content of 15% (solid-state), and a ratio of inoculum to substrate of 2:1 (based on TS). To inhibit the activity of methanogens, we added 19 mmol/L of 2-Bromoethanosulphate (2-BES) in all the test bottles before fermentation. Batch tests for VFA production were carried out using a Batch Test System (Bioprocess Control, Lund, Sweden) with a working volume of 400 ml at 30 °C. The experiment was divided into four groups, named raw substrate, control group, pretreated by *P. sajor-caju* and pretreated by *T. versicolor*. Raw substrate was original solid digestate without autoclave and fungal pretreatment. The control group was autoclaved solid digestate without fungal pretreatment. The group of pretreated by *P. sajor-caju* and *T. versicolor* was solid digestate autoclaved and pretreated by *P. sajor-caju* and *T. versicolor*, respectively.

Samples for the determination of the VFA were extracted from the bottles every two days and analyzed. The mixture samples were poured into centrifuge tubes of 15 ml from each fermentative bottle. After the sample collection, each bottle was sealed with a rubber stopper and flushed nitrogen gas into bottom of bottles for 5 min to replace the oxygen in the headspace, and then connected with Batch Test System again. The collected samples in centrifuge tubes were used for the chemical analysis. All the tests were carried out in triplicate.

2.5. Analytical methods

2.5.1. Determination of ergosterol concentration in fungal biomass

Ergosterol was used as a fungal biomarker. Ergosterol was extracted using an alkaline extraction protocol and determined according to procedure with minor modifications [13]. The modified procedure was as follows: 1.0 g moist sample was put into 15 ml of test tubes with 4 ml 10% KOH in methanol. After 15 min sonication, the test tubes went through a heat treatment (i.e. keeping the tubes in a water bath at 70 °C for 90 min) to release the esterified ergosterol. Subsequently, ergosterol was extracted by adding 1 ml Milli-Q water and 2 ml hexane. The tubes were stirred vigorously for 30 s and centrifuged for 10 min at 4000 r/min, and 1 ml liquid of top phase was transferred into small glass tubes. This step was repeated once more by adding 1 ml hexane for completely removing KOH. After evaporation of the hexane in a water bath at 50 °C in the fume hood for at least a whole night, 1 ml methanol was added into the samples in glass tubes. Afterwards, the samples were sonicated for 4 min and then the liquid was filtered into a vial by using 0.2 μ m syringe filter. Then the ergosterol extracted from samples was analyzed using a high performance liquid

chromatography (HPLC) with C18 column (250 mm × 4.6 mm, Alltech Allsphere ODS-25 μ).

2.5.2. Extracellular enzymatic activity

Extracellular enzymatic activity was analyzed by UV spectrometry (Genesys 105 UV–vis). 1.0–1.5 g sample was suspended at a sample-to-distilled water ratio of 1:20 (w/v) on a shaker at 160 r/min for 30 min. Afterwards, the mixed sample was centrifuged at 12000 × g for 15 min and then the supernatant was filtered using a syringe membrane with 0.45 μm pore size. Laccase activity was measured by the oxidation of 2,6-dimethoxyphenol as described in the study of (Cruz-Morato et al., 2013) [14]. Manganese peroxidase (MnP) activity was measured by monitoring the enzyme's oxidation of Mn (II) to Mn (III) [15].

2.5.3. Fourier transform infrared spectroscopy (FTIR) analysis

Analysis by fourier transform infrared spectroscopy (FTIR) was carried out to determine the functional groups of substrate before and after fungal pretreatment. Prior to the FTIR analyses, the samples were dried in an oven at 105 °C for 8 h and then ground by a cutting mill (Retsch SM 2000, Germany) with a mesh size of 0.2 mm. The prepared samples were analyzed using a Nicolet Impact 400 FTIR spectrometer equipped with a DTGS detector (Perkin–Elmer, the Netherlands). Spectral range was from 4000 to 600 cm⁻¹ with a spectral resolution of 4 cm⁻¹.

2.5.4. Other analysis

TS and VS were determined on a weight basis according to the standard methods (APHA, 2005) [16]. Samples for analysis of soluble chemical oxygen demand (SCOD), ammonium (NH₄⁺-N), phosphate (PO₄³⁻-P) and VFA were first centrifuged at 12000 × g for 15 min with a centrifuge (Thermal Scientific, USA), and then the centrifuges were filtered by syringe membrane with 0.45 μm pore size. Their SCOD, NH₄⁺-N and PO₄³⁻-P were measured using kits (Hach-Lange, Germany) according to Zhang et al. (2016) [17]. The contents of cellulose, hemicellulose and lignin were determined according to the method described by van Soest et al. (1991) using a Fibretherm Fibre Analyzer (Gerhardt, Bonn, Germany) [18]. pH was measured by an Orion 370 PerpHecT Meter (Thermo Fisher Scientific, USA).

The VFA concentration in the filtrate was measured by Gas Chromatography equipped with a 25 m × 320 μm × 0.5 μm Agilent 19091F-112 column and flame ionization detector. Helium was used as carrier gas with a flow rate of 1.8 ml/min. The 0.3 ml filtrate samples were collected in 1.5 ml vial with 1.2 ml internal standard pentanol solutions (320 mg/L), and 10.5 μl 98% H₃PO₄ was added to adjust the pH to 3.5 ± 0.5. The temperature for the injection port, column and detector was set at 240, 240 and 250 °C, respectively.

VFA yield was calculated by Equation (1):

$$\text{VFA yield (mg COD/g VS}_{\text{added}}) = \frac{(\text{VFA}_x - \text{VFA}_i)_x \times V}{\text{VS}_{\text{added}}} \quad (1)$$

where VFA_x represents the VFA concentration in the anaerobic fermentation test (mg COD/L); V represents the volume of fermentation system and VFA_i represents the VFA concentration in inoculum test (mg COD/L); VS_{added} represents the amount of VS of substrate (g) in the test. The conversion factors between individual VFA and COD used in this study were 1.07 g COD/g for acetate, 1.51 g COD/g for propionate, 1.82 g COD/g for butyrate, 2.04 g COD/g for valerate, and 2.20 g COD/g for hexanoate.

3. Results and discussion

3.1. Fermentative VFA production from solid digestate

3.1.1. Effect of fungal pretreatment on VFA yield during anaerobic fermentation

VFA yields in anaerobic fermentation under different conditions are shown in Fig. 1. VFA production in the group with the raw substrate slowly increased during fermentation and the maximum VFA yield obtained was only 107 mg COD/g VS_{added}, because most easily biodegradable components had already been converted to biogas in the methanogenic process in the original digestion plant. The VFA yield of autoclaved sample (110 mg COD/g VS_{added}) was slightly lower than the control group, but the difference was not statistically significant at 95% level. However, the VFA yield from the solid digestate pretreated by *P. sajor-caju* was substantially enhanced and a maximum yield of 240 mg COD/g VS_{added} was obtained at the 14th day of anaerobic fermentation. Evidently, the pretreatment by *P. sajor-caju* significantly improved the anaerobic biodegradability of solid digestate and further increased the VFA yield by approximately 1.17-fold and 1.24-fold compared with that of the control and raw solid digestate, respectively. Over time during fermentation, the VFA yield slightly decreased to 217 mg COD/g VS_{added} from 14th days onwards to the end of fermentation, because the 2-Bromoethanosulphate used for inhibition of methanogens might be consumed or its concentration substantially might reduce, which could lead to VFA consumption and methane production of VFA consumption and conversation into methane.

In contrast, the maximum VFA yield from solid digestate pretreated by *T. versicolor* was only 101 mg COD/g VS after 18 days of anaerobic fermentation, which was 9% and 6% lower compared to the control group and the raw solid digestate, respectively. A comparable result was observed in a previous study [19], which reported that using *T. versicolor* to treat wheat straw resulted in a reduction in biogas production by 20%, compared to the control group (autoclaved but un-inoculated straw). An explanation for the reduction could be that in the presence of *T. versicolor* a higher degradation of cellulose simultaneously occurred than the degradation of lignin during pretreatment [19].

A summarized comparison of VFA yields from different substrates with or without fungal pretreatment is given in Table 2. As shown in Table 2, although the solid digestate used in the present study had been anaerobically digested, the VFA yield from solid

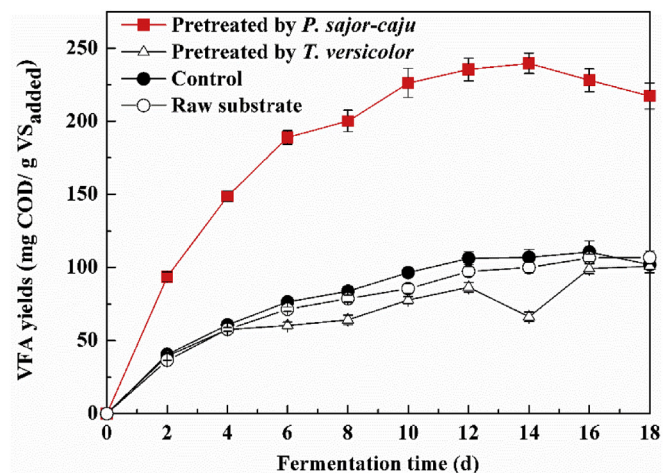


Fig. 1. Effect of fungal pretreatment on VFA yields from solid digestate during anaerobic digestion.

Table 2

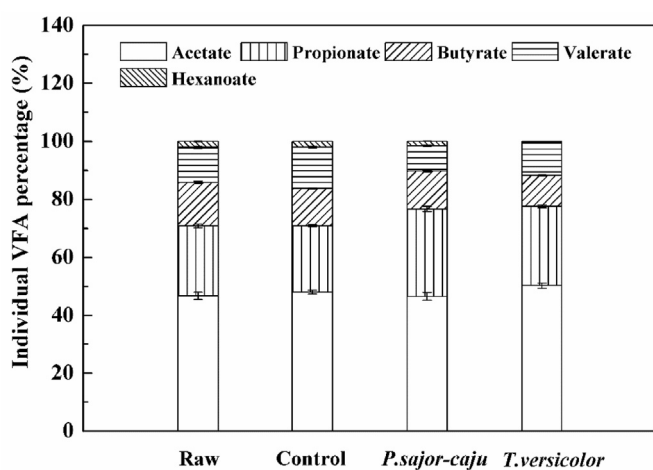
Comparison of VFA yields from solid digestate with fungal pretreatment in this study and other substrates in previous studies.

Substrate	Main Composition			Pretreatment method	Operation condition of anaerobic fermentation	Maximal VFA Yield	Reference
	Cellulose	Hemicellulose	Lignin				
Cattail, <i>Typha latifolia</i>	28.3	11.0	13.5	–	25 °C, control pH = 12	127 mg VFAs/g DM	[21]
Wheat straw	41.1	27.4	24.7	–	35 °C, without pH control	141 mg COD/g TS	[6]
Bagasse	–	–	18.77	Mill and lime pretreatment	55 °C, control pH = 7	360 mg VFAs/g VS	[22]
Waste activated sludge	–	–	–	Thermal pretreatment	35 °C, initial pH = 6	224 mg VFAs/g VS	[23]
Solid digestate	18.1	14.6	31.1	Fungus (<i>P. sajor-caju</i>)	30 °C, without pH control	240 mg COD/g VS	This study

Note: DM = dry matter.

digestate after pretreatment using *P. sajor-caju* was still comparable to that obtained from previous studies, in which the VFA was produced from anaerobic fermentation of different types of raw and pretreated lignocellulosic biomass and even activated sludge. Therefore, fungal pretreatment offers a novel and promising approach to improve the biodegradability of refractory organic materials under anaerobic conditions and further VFA production.

An efficient VFA product chain requires a minimal input of chemicals for pH control. In this study, the initial pH in all tests was in a range of 7.8–8.1. Without pH control, the final pH of mixed broth decreased to 7.1, 6.8, 6.4 and 6.6 after 18-day fermentation with raw digestate, autoclaved digestate, *P. sajor-caju* pretreated digestate and *T. versicolor* pretreated digestate as substrate, respectively. Apparently, the lowest pH was obtained from fermented sample pretreated by *P. sajor-caju* mainly because of higher VFA production. Besides, the pH values were always above the limited value of 6.0 for anaerobic fermentation of lignocellulosic biomass during the whole fermentation process [20]. Thus, it is not necessary to dose chemicals to control the pH for comparable VFA yield from solid digestate with fungal pretreatment.

**Fig. 2.** Effect of fungal pretreatment on VFA profile by the end of anaerobic fermentation.**Table 3**NH₄⁺-N and PO₄³⁻-P release after anaerobic fermentation of solid digestate pretreated by *P. sajor-caju* and *T. versicolor*.

Samples	NH ₄ ⁺ -N concentration (mg/L)	PO ₄ ³⁻ -P concentration (mg/L)	NH ₄ ⁺ -N release (mg/g VS _{added})	PO ₄ ³⁻ -P release (mg/g VS _{added})
Raw substrate	1067 ± 21	314.0 ± 2.4	9.34 ± 1.31	1.13 ± 0.17
Autoclaved	1056 ± 18	328.0 ± 3.8	8.37 ± 0.83	0.92 ± 0.21
Pretreated by <i>P. sajor-caju</i>	1121 ± 30	336.0 ± 4.0	13.88 ± 1.78	1.59 ± 0.29
Pretreated by <i>T. versicolor</i>	1085 ± 19	334.0 ± 1.8	10.80 ± 0.98	1.42 ± 0.36

3.1.2. Effect of fungal pretreatment on VFA profile

The profiles of individual VFAs from raw, autoclaved (control group) and pretreated substrates were analyzed and are shown in Fig. 2. Acetate was the most prevalent VFA products at all tests, accounting for more than 47% by the end of fermentation, followed by propionate of up to 23%. Butyrate and valerate was respectively below 15% and 14% of the total VFA products. Whereas, hexanoate took a fraction less than 2% and was the lowest VFA fraction in all tests. After the substrate was pretreated by *P. sajor-caju* and *T. versicolor*, the propionate content increased to 30% and 27%, respectively, and valerate content decreased, compared to the control group. The results can be probably explained by reduction in chain elongation during fermentation process [24]. Compared to raw substrate, acetate content in mixed VFA products with fungal pretreatment by *P. sajor-caju* and *T. versicolor* only decreased 0.2% and increased 3.0%, respectively. Hence, the total acetate and propionate were dominant VFA species in any investigated tests, with a total range of more than 70% by the end of fermentation. Specifically, the total contents of acetate and propionate increased to 78%, higher about 7% than that of raw solid digestate. The high content of micromolecular organic acids in the produced VFA mixtures indicates that the proposed pretreatment method might be suitable and potentially beneficial for possible subsequent bioprocesses, such as downstream bio-refinery and/or biological nutrient removal processes in WWTPs [25].

3.1.3. Ammonium and phosphate release after anaerobic fermentation

Pretreatment with *P. sajor-caju* significantly enhanced the VFA production from solid digestate, however, a number of ammonium and phosphate were released from organic matters during anaerobic fermentation of solid digestate (as shown in Table 3). The NH₄⁺-N concentration at all tests was in a range of 1067–1121 mg/L after 18-day anaerobic fermentation, corresponding to a NH₄⁺-N release of 9.34–13.88 mg/g VS_{added}. The PO₄³⁻-P concentration at all tests was in a range of 314–336 mg/L, corresponding to a PO₄³⁻-P release of 0.92–1.59 mg/g VS_{added}. For the purpose of maximizing the efficient application of VFAs and resource recovery, the NH₄⁺-N and PO₄³⁻-P are suggested to be separated from the fermentation broth. Various technologies may be employed to recover the resources from the fermentation broth, such as ammonium stripping or the formation of struvite as a fertilizer [26], but the influence of fermentation broth characteristics should be further detailedly

investigated.

3.2. Why did fungal pretreatment influence VFA production of solid digestate?

3.2.1. Fungi growth during fungal pretreatment

A good colonization is a prerequisite for an effective fungal pretreatment of lignocellulosic materials. Ergosterol has been widely used to quantify fungal biomass in soil or organic matters because it is an important component in the cellular membrane of almost all fungi, which was not commonly produced by other organisms [27]. Thus, in this study, ergosterol content was employed to quantify the fungal biomass growth of each species. As shown in Fig. 3, both fungi used in the present study were able to grow on the substrate. As shown in Fig. S1, visual observation also confirmed the growth of *P. sajor-caju* and *T. versicolor* in solid digestate. *P. sajor-caju* grew well on substrate in the first 2 weeks of inoculation and a substantial increase of *P. sajor-caju* mycelia was observed. However, a slight decrease in the fungal biomass was observed from week 2 to week 4 of the inoculation. In the present study, ergosterol content of *P. sajor-caju* after 6 weeks was 225 mg/kg VS, which was obviously higher than the ergosterol content (120 mg/kg DM) for the same fungus specie (*P. ostreatus*) with wheat straw as substrate [13]. The higher ergosterol content might result from the difference in culture substrates. The ergosterol content of *T. versicolor* in treated substrate had a different pattern. It is only first week that a substantial increase in ergosterol content of *T. versicolor* was observed. Afterwards, the ergosterol content of *T. versicolor* decreased from 111 to 26 mg/kg VS during the second week, and further slowly increased to 54 mg/kg VS by the end of 6 weeks of inoculation. Different growth stages could influence ergosterol content in fungi, but the content is not always positively related with degradation of components in substrate [13,28].

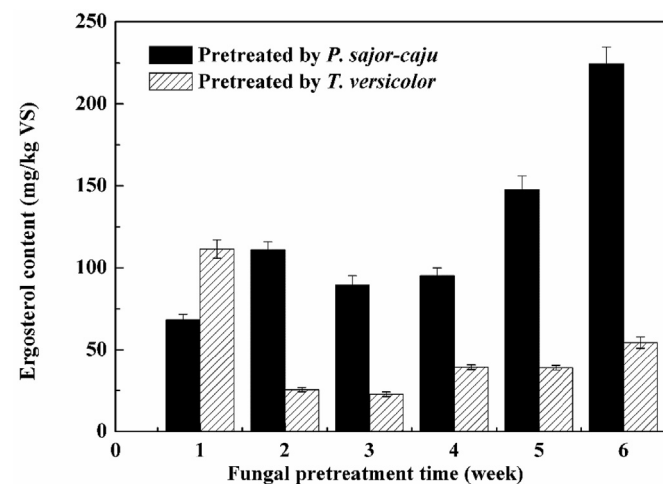


Fig. 3. Ergosterol content in solid digestate during *P. sajor-caju* and *T. versicolor* pretreatment.

3.2.2. Effect of fungal pretreatment on chemical composition of solid digestate

Fungal growth on substrate is accompanied by degradation of organic matter. Therefore, the effects of fungal pretreatment on the chemical composition of solid digestate was studied and presented in Table 4. The autoclave process had a significant impact on the composition of solid digestate. The sum of cellulose, hemicellulose and lignin content in solid digestate (dry substrate) increased from 63.8% to 83.6% after autoclave process, which indicated that some ash fractions in solid digestate were removed or dissolved.

The lowest lignin content of 22.4% was observed in *P. sajor-caju* after 6 weeks of inoculation, which was about 38% and 28% lower than the autoclaved and raw substrate, respectively. The lignin content in solid digestate after the pretreatment by *T. versicolor* declined only to 27.5%. It means that compared to *T. versicolor*, *P. sajor-caju* had a stronger efficiency in degradation of lignin in solid digestate. The fungal pretreatment especially by *P. sajor-caju* could break lignin-carbohydrate linkage and release more cellulose and hemicellulose from the complex structure of solid digestate, which will be beneficial to anaerobic fermentation because cellulose is an easy degradable substrate for anaerobic microbes [29]. Comparing compositions of solid digestate after the pretreatment by *P. sajor-caju* with those of the raw substrate, the cellulose content increased from 18.1% to 34.1%. The higher cellulose content could be explained by the smaller loss of cellulose relative to the loss of other polymers in substrate. However, *T. versicolor* presented a lower ability of lignin degradation and a higher ability of cellulose degradation than *P. sajor-caju*, resulting in no improvement in the anaerobic fermentation of solid digestate [19]. As shown in Fig. 4 and Table S1, the peaks around at 2922 cm⁻¹ corresponding to C–H stretching in methyl and methylene groups in lignin decreased with *P. sajor-caju* [30], while the intensity of the peaks around 1300 cm⁻¹ that are assigned to the C–O–C groups in

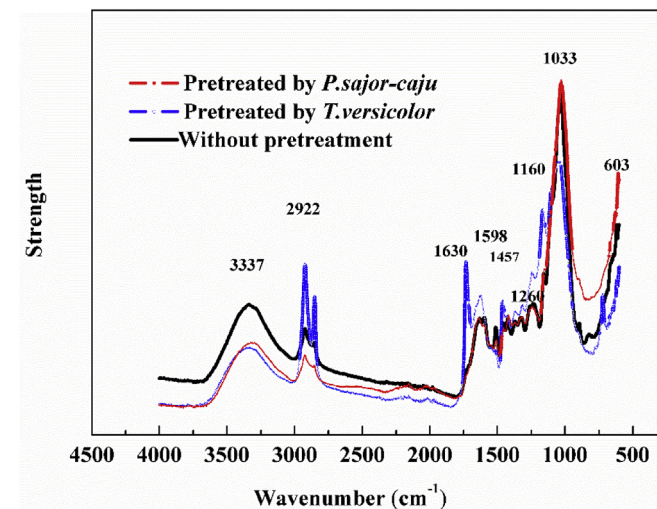


Fig. 4. FTIR spectra of solid digestate with fungal pretreatment for 6 weeks and without pretreatment.

Table 4
Lignocellulosic composition of solid digestate before and after 6 weeks of fungal pretreatment.

Substrate	Cellulose (% dry weight)	Hemicellulose (% dry weight)	Lignin (% dry weight)	Cellulose/lignin ratio
Raw substrate	18.1 ± 0.3	14.6 ± 0.5	31.1 ± 0.4	0.58
Control (autoclaved)	31.1 ± 0.8	17.6 ± 0.3	34.9 ± 0.7	0.89
Pretreated by <i>P. sajor-caju</i>	34.1 ± 0.9	13.6 ± 0.2	22.4 ± 0.2	1.52
Pretreated by <i>T. versicolor</i>	26.9 ± 0.5	14.5 ± 0.5	27.5 ± 0.3	0.98

cellulose and hemicellulose did not substantially change after 6 weeks inoculation [31]. On the other hand, the fungal pretreatment did not noticeably change the hemicellulose content in the substrate, compared with cellulose and lignin content.

The cellulose/lignin ratio is a useful parameter to evaluate the biodegradability of substrate [32,33]. In the present study, a maximum cellulose/lignin ratio of 1.52 was observed after pretreatment by *P. sajor-caju*. The results indicated that the lignin was more degraded than the cellulose during *P. sajor-caju* pretreatment, presumably implying the *P. sajor-caju* preferred the lignin degradation rather than cellulose utilization. Furthermore, the fungal pretreatment using *P. sajor-caju* selectively degraded lignin, thus de-encrusting the cellulosic fibers and enhancing the availability of binding sites of feedstock for hydrolytic enzymes during anaerobic fermentation [34,35]. These results were the generally positive reasons for the significant improvement of VFA production from the solid digestate pretreated by *P. sajor-caju* (Fig. 1). The similar preference of *P. sajor-caju* to lignin when grown on lignocellulosic biomass has been previously confirmed by other studies, who concluded that the higher lignin removal was positively related with better anaerobic digestion in terms of biogas production [36]. The low cellulose/lignin ratio of solid digestate pretreated by *T. versicolor* demonstrated that *T. versicolor* degraded more cellulose simultaneously with the lignin degradation, which resulted in a low cellulose recovery [19].

3.2.3. Ligninolytic enzymatic activities in solid digestate during fungal pretreatment

Fig. 5 depicts activities of two dominant ligninolytic enzymes in the substrate during the fungal pretreatment. However, lignin peroxidase was not detected in the fungal pretreatment system, which might be attributed to that *Pleurotus* species (such as, *P. sajor-caju*) are not able to secrete lignin peroxidase. Different levels of laccase and manganese peroxidase (MnP) activity were detected after one week of inoculation, which has not been reported previously in the literature on degradation of solid digestate. *T. versicolor* exhibited relatively high laccase activity and rapidly increased in the first week and then reached the highest value of 284.9 U/g VS after two weeks of pretreatment (Fig. 5(a)), which could oxidize phenolic rings to phenoxyl radicals. It is particularly interesting that appearance of laccase activity in fungal inoculations has been correlated with fungal growth in many cases [37]. In this study, a fast growth of *T. versicolor* was recorded with a corresponding increase in laccase activity during the first week of inoculation, which might lead to a high degradation of lignin. Similarly, the result was in accordance with a previous study correlating the high production of laccase and lignin degradation by *T. versicolor* [38]. However, laccase activity rapidly decreased by inactivation or proteolysis as inoculation developed fruit bodies, which was achieved by

adjusting nutritional demands of fungal mycelia [39]. In contrast, the laccase activity in *P. sajor-caju* increased slowly and only reached a maximum value of 45.8 U/g VS after 4 weeks of inoculation.

As shown in Fig. 5(b), the MnP activity in *P. sajor-caju* related with phenolic and non-phenolic lignin degradation was much higher than that in *T. versicolor* during the 6 weeks incubation. The MnP activity in *P. sajor-caju* substantially increased in the first week and gradually increased to a maximum of 103.1 U/g VS, which was above 2 folds than that in *T. versicolor* obtained in the 5th week. The high MnP activity positive correlated with the rapid decline in pH (and further high VFA production) from *P. sajor-caju* because the production of some low molecular acids such as oxalate acid and alkylitaconic acids were associated with MnP activity as a chelator of produced Mn^{3+} [40]. Furthermore, some of those acids such as alkylitaconic acids could inhibit hydroxyl radicals that were released with lignin degradation to attack cellulose [41]. It is worthy noticing due to low redox potential of laccases, laccase only degrade low-redox potential compounds and not allows the oxidation of more recalcitrant aromatic compounds compared to MnP [38], so, the *P. sajor-caju* showed a stronger ability of delignification than *T. versicolor*, which agreed the results of higher cellulose and lower lignin content of solid digestate after fungal pretreatment by *P. sajor-caju* (Table 4). Above all, it is evident that the two different fungal strains presented different degradation pathways with the same substrate. The dominant function enzyme for delignification by *P. sajor-caju* was MnP, while the laccase was dominant ligninolytic enzyme in *T. versicolor*. But the laccase not only had a weaker ability of degradation of lignin than MnP, but also some laccase could absorb and bind to cellulose, which might limit the hydrolysis of cellulose by various cellulases [42], and negatively affect subsequent anaerobic fermentation for VFA production.

3.2.4. Microstructure change

The morphology of raw, autoclaved and pretreated substrate is shown in Fig. 6. For raw substrate, it had a compact structure with small holes in surface, which could be resulted in the original digestion process, but the surface maintained almost integrity. Autoclave process led to a slightly crack and rough. Fig. 6(c) indicated that a large amount of mycelia penetrated the lignocellulosic structure of solid digestate and the surface of substrates was substantially damaged. Apparently, the *P. sajor-caju* was more effective in damaging the solid digestate structure through lignin degradation, leading to increases in surface area and pore size of substrate. The *P. sajor-caju* pretreatment promoted the accessibility of anaerobic microbes to the favored cellulose and would simultaneously allow enzymes to further decompose the cellulose and improve the anaerobic digestion processes as shown in Fig. 1 [43]. However, the damage of the solid digestate structure by *T. versicolor*

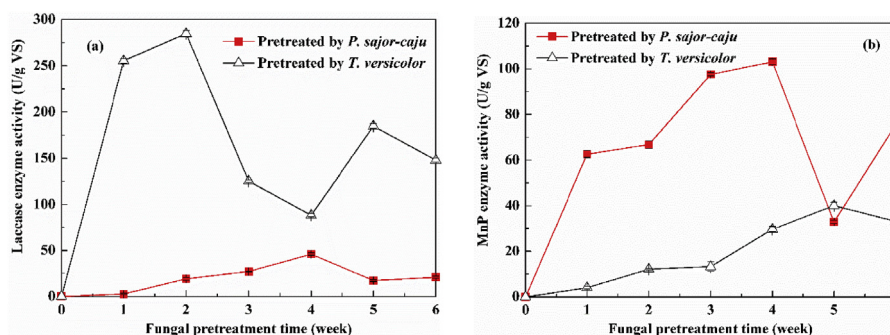


Fig. 5. Enzymatic activities during the pretreatment by two fungi: (a) laccase activity; (b) MnP activity.

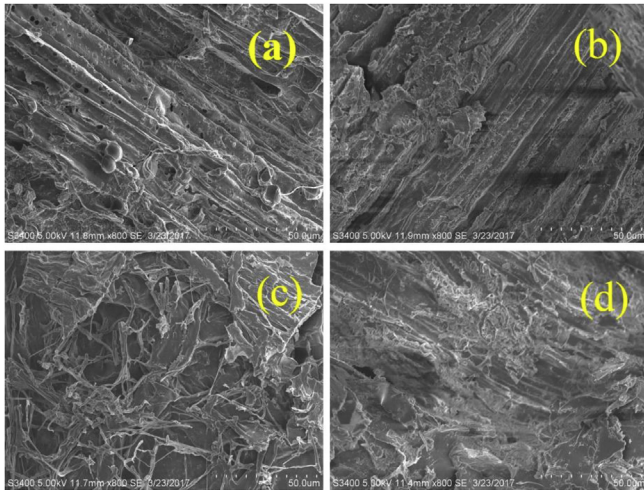


Fig. 6. SEM images of solid digestate (a) raw, (b) after autoclaving, (c) pretreated by *P. sajor-caju*, and (d) pretreated by *T. versicolor*.

was weaker than that by *P. sajor-caju* as shown in Fig. 6(d) and there was not enough surface area for enzyme reaction, which might be the reason that the VFA production from the solid digestate by *T. versicolor* was not enhanced.

3.2.5. pH variation during fungal pretreatment of solid digestate

pH is an important parameter that can significantly impact fungal growth and production of non-specific extracellular enzymes [37]. The pH variations in substrate during fungal pretreatment are shown in Fig. 7. In this study, solid digestate presented an initial pH ranging from 6.6 to 6.9, but the pH dramatically decreased by about one unit during the first week. Afterwards, the pH in *P. sajor-caju* and *T. versicolor* fluctuated slightly, and then slowly decreased and stabilized at 4.9 and 4.7, respectively, by the end of the pretreatment trial. The general pH profile was similar to a previous study of Dong (et al., 2013), who observed a pH decrease from 5.5 to 3.2 during the growth of *Pleurotus ostreatus* on sugarcane bagasse for three weeks [44]. The decrease in pH might be attributed to the production of organic acids, such as carboxylic and alkylitaconic acids, from the degradation of lignin and hemicellulose by white rot fungi [45]. Chen (et al., 1983)

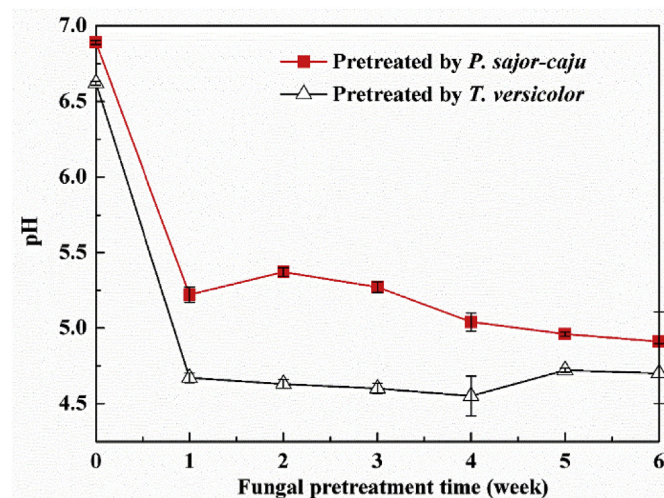


Fig. 7. pH in substrate during *P. sajor-caju* and *T. versicolor* pretreatment.

investigated the breakdown of spruce wood lignin by typical white rot fungi (*P. chrysosporium*) and stated that more than 35% of the products with low molecular weight were aromatic carboxylic acids. Therefore, the pH variation may be used as an indicator of the degradation of solid digestate [46].

4. Conclusions

A novel pretreatment method using white rot fungi to improve solid-state fermentative VFA production from solid digestate was examined and successfully developed. The present study demonstrated the fungal pretreatment using *P. sajor-caju* during 6 weeks of inoculation substantially enhanced the VFA yield by 1.17-fold and 1.24-fold, compared with the control group with autoclaved substrate and the group with raw substrate, respectively. The VFA yield of 240 mg COD/g VS from solid digestate pretreated by *P. sajor-caju* was comparable to the reported data from different biomass without anaerobic digestion before. In contrast, the pretreatment using *T. versicolor* slightly decreased the VFA yield. The mechanisms analysis indicated that fungal pretreatment using *P. sajor-caju* effectively damaged the structure and changed the characteristics of solid digestate owing to the secretion of ligninolytic enzymes for lignin degradation. Moreover, the improved VFA yields were also linked to the increases in surface area and pore size of the solid digestate and selective or favorable de-encrustation/delignification. Therefore, the fungal pretreatment using *P. sajor-caju* is a potential and feasible approach to enhance the degradability of solid digestate and conversion of solid digestate into high value added products, such as VFAs. However, the fungal pretreatment should be further optimized when applied to the utilization of solid digestate because of the long fungal pretreatment. Additionally, it is interesting to further investigate efficient fungal strains which are preferential to delignification during pretreatment of different solid digestate. Suitable and efficient fungal strains should be selected according to the characteristics of substrate and required degradation degree of different components of biomass.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.energy.2018.08.082>.

References

- [1] Khan MA, Ngo HH, Guo WS, Liu Y, Nghiem LD, Hai FI, et al. Optimization of process parameters for production of volatile fatty acid, biohydrogen and methane from anaerobic digestion. *Bioresour Technol* 2016;219:738–48.
- [2] Zhou AJ, Guo ZC, Yang CX, Kong FY, Liu WZ, Wang AJ. Volatile fatty acids productivity by anaerobic co-digesting waste activated sludge and corn straw: effect of feedstock proportion. *J Biotechnol* 2013;168:234–9.
- [3] Azman S, Khadem AF, van Lier JB, Zeeman G, Plugge CM. Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. *Crit Rev Environ Sci Technol* 2015;45:2523–64.
- [4] Tambone F, Scaglia B, D'Imporzano G, Schievano A, Orzi V, Salati S, et al. Assessing amendment and fertilizing properties of digestates from anaerobic

- digestion through a comparative study with digested sludge and compost. *Chemosphere* 2010;81:577–83.
- [5] Behera S, Arora R, Nandhagopal N, Kumar S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renew Sustain Energy Rev* 2014;36:91–106.
 - [6] Motte J-C, Sambusiti C, Dumas C, Barakat A. Combination of dry dark fermentation and mechanical pretreatment for lignocellulosic deconstruction: an innovative strategy for biofuels and volatile fatty acids recovery. *Appl Energy* 2015;147:67–73.
 - [7] Liu HB, Xiao H, Yin B, Zu YP, Liu H, Fu B, et al. Enhanced volatile fatty acid production by a modified biological pretreatment in anaerobic fermentation of waste activated sludge. *Chem Eng J* 2016;284:194–201.
 - [8] Shirkavand E, Baroutian S, Gapes DJ, Young BR. Pretreatment of radiata pine using two white rot fungal strains *Stereum hirsutum* and *Trametes versicolor*. *Energy Convers Manag* 2017;142:13–9.
 - [9] Marshall CW, LaBelle EV, May HD. Production of fuels and chemicals from waste by microbiomes. *Curr Opin Biotechnol* 2013;24:391–7.
 - [10] Oehmen A, Lemos PC, Carvalho G, Yuan ZG, Keller J, Blackall LL, et al. Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res* 2007;41:2271–300.
 - [11] Garoma T, Pappaterra D. An investigation of ultrasound effect on digestate solubilization and methane yield. *Waste Manag* 2018;71:728–33.
 - [12] Yue Z, Teater C, Liu Y, Maclellan J, Liao W. A sustainable pathway of cellulosic ethanol production integrating anaerobic digestion with biorefining. *Bio-technol Bioeng* 2010;105:1031–9.
 - [13] van Kuijk SJA, Sonnenberg ASM, Baars JJP, Hendriks WH, Cone JW. Fungal treatment of lignocellulosic biomass: importance of fungal species, colonization and time on chemical composition and in vitro rumen degradability. *Anim Feed Sci Technol* 2015;209:40–50.
 - [14] Cruz-Morató C, Ferrando-Climent L, Rodríguez-Mozaz S, Barceló D, Marco-Urrea E, Vicent T. Degradation of pharmaceuticals in non-sterile urban wastewater by *Trametes versicolor* in a fluidized bed bioreactor. *Water Res* 2013;47:5200–10.
 - [15] Paszczyński A, Crawford RL, Huynh V-B. Manganese peroxidase of *Phanerochaete chrysosporium*: purification. *Methods in enzymology*. Academic Press; 1988. p. 264–70.
 - [16] APHA. Standard methods for the examination of water and wastewater. DC USA: Am. Public Health Assoc. APHA Wash.; 2005.
 - [17] Zhang X, Tao Y, Hu J, Liu G, Spanjers H, van Lier JB. Biomethanation and microbial community changes in a digester treating sludge from a brackish aquaculture recirculation system. *Bioresour Technol* 2016;214:338–47.
 - [18] van Soest PV, Robertson J, Lewis B. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 1991;74:3583–97.
 - [19] Tuyen VD, Cone JW, Baars JJP, Sonnenberg ASM, Hendriks WH. Fungal strain and incubation period affect chemical composition and nutrient availability of wheat straw for rumen fermentation. *Bioresour Technol* 2012;111:336–42.
 - [20] Asanuma N, Hino T. Tolerance to low pH and lactate production in rumen bacteria. *Anim Sci Technol* 1997;68:367–76.
 - [21] Chen Y, Wen Y, Zhou JW, Xu C, Zhou Q. Effects of pH on the hydrolysis of lignocellulosic wastes and volatile fatty acids accumulation: the contribution of biotic and abiotic factors. *Bioresour Technol* 2012;110:321–9.
 - [22] Rughoonundun H, Mohee R, Holtzapple MT. Influence of carbon-to-nitrogen ratio on the mixed-acid fermentation of wastewater sludge and pretreated bagasse. *Bioresour Technol* 2012;112:91–7.
 - [23] Liu XL, Liu H, Chen JH, Du GC, Chen J. Enhancement of solubilization and acidification of waste activated sludge by pretreatment. *Waste Manag* 2008;28:2614–22.
 - [24] Agler MT, Wrenn BA, Zinder SH, Angenent LT. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. *Trends Biotechnol* 2011;29:70–8.
 - [25] Tong J, Chen Y. Recovery of nitrogen and phosphorus from alkaline fermentation liquid of waste activated sludge and application of the fermentation liquid to promote biological municipal wastewater treatment. *Water Res* 2009;43:2969–76.
 - [26] Huang WW, Huang WL, Yuan T, Zhao ZW, Cai W, Zhang ZY, et al. Volatile fatty acids (VFAs) production from swine manure through short-term dry anaerobic digestion and its separation from nitrogen and phosphorus resources in the digestate. *Water Res* 2016;90:344–53.
 - [27] Bååth E. Estimation of fungal growth rates in soil using 14 C-acetate incorporation into ergosterol. *Soil Biol Biochem* 2001;33:2011–8.
 - [28] Gao Y, Chen T, Breuil C. Ergosterol - a measure of fungal growth in wood for staining and pitch control fungi. *Bioresour Technol* 1993;7:621–6.
 - [29] Wan CX, Li YB. Fungal pretreatment of lignocellulosic biomass. *Biotechnol Adv* 2012;30:1447–57.
 - [30] Gao Z, Fan Q, He Z, Wang Z, Wang X, Sun J. Effect of biodegradation on thermogravimetric and chemical characteristics of hardwood and softwood by brown-rot fungus. *Bioresour Technol* 2016;211:443–50.
 - [31] Yang X, Zeng Y, Ma F, Zhang X, Yu H. Effect of biopretreatment on thermogravimetric and chemical characteristics of corn stover by different white-rot fungi. *Bioresour Technol* 2010;101:5475–9.
 - [32] Lalak J, Kasprzycka A, Martyniak D, Tys J. Effect of biological pretreatment of *Agropyron elongatum* 'BAMAR' on biogas production by anaerobic digestion. *Bioresour Technol* 2015;200:194–200.
 - [33] Zhong W, Zhang Z, Luo Y, Sun S, Qiao W, Xiao M. Effect of biological pretreatments in enhancing corn straw biogas production. *Bioresour Technol* 2011;102:11177–82.
 - [34] Vermaas JV, Petridis L, Qi X, Schulz R, Lindner B, Smith JC. Mechanism of lignin inhibition of enzymatic biomass deconstruction. *Biotechnol Biofuels* 2015;8:217.
 - [35] Zhao J, Zheng Y, Li Y. Fungal pretreatment of yard trimmings for enhancement of methane yield from solid-state anaerobic digestion. *Bioresour Technol* 2014;156:176–81.
 - [36] van Kuijk SJA, Sonnenberg ASM, Baars JJP, Hendriks WH, Cone JW. Fungal treated lignocellulosic biomass as ruminant feed ingredient: a review. *Biotechnol Adv* 2015;33:191–202.
 - [37] Tavares A, Coelho M, Agapito M, Coutinho J, Xavier A. Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. *Appl Biochem Biotechnol* 2006;134:233–48.
 - [38] Baldrian P. Fungal laccases - occurrence and properties. *FEMS Microbiol Rev* 2006;30:215–42.
 - [39] Moore D, Robson GD, Trinci AP. 21st century guidebook to fungi with CD. Cambridge University Press; 2011.
 - [40] Kishi K, Wariishi H, Marquez L, Dunford HB, Gold MH. Mechanism of manganese peroxidase compound II reduction. Effect of organic acid chelators and pH. *Biochem* 1994;33:8694–701.
 - [41] Rahmawati N, Ohashi Y, Watanabe T, Honda Y, Watanabe T. Ceriporic acid B, an extracellular metabolite of *Ceriporiopsis subvermisporea*, suppresses the depolymerization of cellulose by the fenton reaction. *Biomacromolecules* 2005;6:2851–6.
 - [42] Kiiskinen L-L, Palonen H, Linder M, Viikari L, Kruus K. Laccase from *Melanocarpus albomyces* binds effectively to cellulose. *FEBS Lett* 2004;576:251–5.
 - [43] Talebnia F, Karakashev D, Angelidaki I. Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation. *Bioresour Technol* 2010;101:4744–53.
 - [44] Dong XQ, Yang JS, Zhu N, Wang ET, Yuan HL. Sugarcane bagasse degradation and characterization of three white-rot fungi. *Bioresour Technol* 2013;131:443–51.
 - [45] Bugg TD, Ahmad M, Hardiman EM, Rahmanpour R. Pathways for degradation of lignin in bacteria and fungi. *Nat Prod Rep* 2011;28:1883–96.
 - [46] Chen CL, Chang H, Kirk TK. Carboxylic-acids produced through oxidative cleavage of aromatic rings during degradation of lignin in spruce wood by *Phanerochaete-Chrysosporium*. *J Wood Chem Technol* 1983;3:35–57.