Degradation of Lignocellulosic Components in Un-pretreated Vinegar Residue Using an Artificially Constructed Fungal Consortium

Yaoming Cui, Xiaofang Dong,* Jianming Tong, and Song Liu

The objective of this work was to degrade lignocellulosic components in un-pretreated vinegar residue (VR) using a fungal consortium. Consortium-29, consisting of *P. chrysosporium*, *T. koningii*, *A. niger*, and *A. ficuum* NTG-23, was constructed using orthogonal design combined with two-way interaction analysis. After seven days of cultivation, the reducing sugar yield reached 35.57 mg per gram of dry substrate (gds^{-1}), which was 108.01% higher than the control (17.10 mg gds^{-1}). Additionally, the xylanase and CMCase activity reached 439.07 U gds^{-1} and 8.15 U gds^{-1}, which were 432.08% and 243.88% higher than that of pure cultures of *A. niger* (82.52 U gds^{-1}) and *P. chrysosporium* (2.37 U gds^{-1}), respectively. The cellulose, hemicellulose, and lignin contents decreased by 17.11%, 68.61%, and 14.44%, respectively, compared with that of the raw VR. The optimal fermentation conditions of consortium-29 were as follows: incubation temperature 25 °C, initial pH 6, initial moisture content 70%, inoculum size 1×10^6 spores/mL, incubation time 5 days, urea/VR 1%, and $MnSO_4 \cdot H_2O$/VR 0.03%. This study suggests that consortium-29 is an efficient fungal consortium for un-pretreated VR degradation and has a potential application in lignocellulosic waste utilization with a low cost of operation.

Keyword: Lignocellulose degradation; Un-pretreated vinegar residue; Fungal consortia; Culture conditions optimization

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INTRODUCTION

Vinegar residue (VR), a waste byproduct, is generated during the process of vinegar production by solid state fermentation (SSF) (Wang *et al*. 2011), and more than 2 million tons of VR are generated annually in China. VR is of poor nutritive value and low digestibility because of its high content of lignocellulosic components (Song *et al*. 2013). In fact, it is estimated that 10 to 50 billion tons of lignocellulosic materials per year, accounting for about 50% of the biomass in the world, could be sustainably harvested (Claassen *et al*. 1999). The renewability of lignocellulosic materials makes them the focus of high-level research because of the enormous economic, environmental, and social benefits.

In recent studies, lignocellulosic resources have been used to produce value-added products, including biofuels, biopolymers, chemicals, fertilizer, and animal feeds (Yang *et al*. 2004; Kausar *et al*. 2010; Zhang *et al*. 2011; Kalyani *et al*. 2013). The microbial strategy has attracted more and more attention due to the simplicity and low capital investment (Yang *et al*. 2011). The critical step in all the bioconversion processes is the degradation of recalcitrant carbohydrate polymers into monomer sugar.

As the low efficiency is the primary hindrance in current biodegradation processes, researchers have been paying more attention to fungi in order to improve the degradation efficiency. Fungi are efficient degraders because of their extracellular enzymatic system, hyphal penetration power, and the ability to produce an enormous number of spores, which can invade substrates quickly (Kausar *et al*. 2010; Sharma and Arora 2013). Because no single strain could produce all the enzymes necessary for lignocellulose degradation, current research is focused on fungal consortia with advantages of avoiding feedback regulation and metabolite repression (Wongwilaiwalin *et al*. 2010). Compared with traditional pure culture fermentations, mixed culture fermentations can allow the use of cheap and impure wastes, overcome the limitation of nutrition, achieve higher product yield and growth rate, and strengthen the protection of the culture from contamination (Yang *et al*. 2004; Lin *et al*. 2011). Although traditional fungal consortia obtained directly from nature usually have strong lignocellulose degradation abilities, their capabilities are easy to degenerate because of their unknown and complicated composition, adversely affecting the potential for practical application (Kato *et al*. 2005; Wongwilaiwalin *et al*. 2010). Since more and more fungi capable of lignocellulose degradation have been isolated and purified, there are increased opportunities for artificial construction of fungal consortia to improve the degradation efficiency.

Although the decomposition of lignocellulosic waste has been studied extensively, almost all of these studies involve a variety of pretreatment methods, such as acid hydrolysis, alkaline hydrolysis, and steam explosion. These strategies can separate lignin from polysaccharides, demolish the special crystal structure, and break down cellulose, leading to a high degradation rate with a relatively short period of incubation. However, the high concentrations of acids are corrosive, toxic, and hazardous, and need costly equipment that can resist corrosion (Yang and Wyman 2008). Alkaline treatment is a relatively slow process, and the added alkali must be removed (Bjerre *et al*. 1996; Chang *et al*. 2001). The steam explosion process involving the application of high-pressure steam consumes a great deal of energy (Mosier *et al*. 2005). These processes also generate various by-products, such as phenolic compounds, furfurals, and organic acids (Palmqvist and Hahn-Hägerdal 2000; Panagiotou and Olsson 2007), which adversely affect the growth of microorganisms in the subsequent fermentation. Both the pretreatment process and removal of toxic compounds mean high costs and significant environmental risks.

By contrast, no toxic compounds are usually detected in the fermented products without pretreatment (Jwanny *et al*. 1995; Karunanandaa *et al*. 1995; Adamović *et al*. 1998). However, there were very few reports on the biodegradation of un-pretreated lignocellulosic materials. Moreover, these studies did not adopt solid-state fermentation (SSF).

Therefore, the aim of this work was to degrade the lignocellulose in un-pretreated VR using an artificial constructed fungal consortium by SSF. This study will help generate a better understanding of the advantages of consortia over pure cultures. It may also provide the experimental basis for highly efficient and cost-competitive decomposition of lignocellulose, which could have a significant impact on the understanding of lignocellulosic waste utilization.

EXPERIMENTAL

Microorganisms

Aspergillus niger ACCC 30557, *Trichoderma viride* ACCC 30552, and *Phanerochaete chrysosporium* ACCC 30414 were purchased from the Agricultural Culture Collection of China (ACCC). *Trichoderma koningii* CGMCC 3.2878 and *Candida utilis* CGMCC 2.1180 were obtained from the China General Microbiological Culture Collection Center (CGMCC). *Aspergillus ficuum* NTG-23 was a mutant strain acquired by our lab from *Aspergillus ficuum* CGMCC 3.4322 (Wang *et al.* 2011). Spore suspensions were prepared for these fungi by growing them on malt-agar, Czapek's, and potato dextrose agar at 28 °C for two weeks. Then, the spores were washed off with sterile saline and scattered with glass beads for 30 min. The final spore concentration of the working spore suspensions was adjusted to 1×10^7 spores/mL.

Substrate and SSF

VR was obtained from the Shanxi province in China and was air-dried and utilized as a substrate for SSF without being milled. The chemicals (g/L) of Mandel's medium (Lin *et al*. 2011) with minor modifications were as follows: polysorbate 80 (Tween-80®) 2, $NaNO_3$ 2, KH_2PO_4 1.5, $CaCl_2$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.005, $MnSO_4 \cdot H_2O$ 0.0016, $ZnSO_4 \cdot 7H_2O$ 0.0014, and $CoCl_2 \cdot 6H_2O$ 0.0005; the pH was adjusted to 6. The mineral medium (45 mL) was added to 30 g of well mixed VR containing 0.3 g of urea. After sterilization for 20 min at 121 °C in a 500-mL Erlenmeyer flask, 1 mL of spore suspension (1×10^7 spores/mL) was aseptically added to the substrate and mixed thoroughly. The incubation temperature was 28 °C, and the incubation time was limited to 7 days.

Sugar Extraction and Determination

Reducing sugar was extracted by suspending the fermented products in buffer and shaking for 4 h at 250 rpm. Following this, the mixture was separated by centrifugation (3,000 rpm for 3 min) to obtain a culture filtrate. The clarified supernatant was then collected and used as the source of reducing sugar and enzymes. Lignocellulose degradation capacity in the supernatant was investigated based on the amount of released reducing sugar, which was quantified colorimetrically as glucose equivalent using the 3, 5-dinitrosalicylic acid (DNS) method (Miller 1959).

Enzyme Assay

Cellulase activity was detected using sodium carboxymethyl cellulose (CMC) as the substrate, which is a soluble cellulose derivative, according to the method of Feng *et al*. (2011). Xylanase activity was tested by the methodology described by Latif *et al*. (2006). β-glucanase activity was assayed using dextran from leuconostoc as the substrate (Li *et al*. 2009). α-amylase activity was estimated by measuring the maltose content using the method of DNS (Dahlqvist 1962).

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of product from the substrates in 0.1 M acetic buffer at pH 5.50 (0.05 M phosphate buffer, pH 6.90 for α-amylase) per min at 37 °C. The activities of the enzyme were expressed as units per gram of dry substrate (U gds^{-1}).

Construction of Consortia using Orthogonal Design

A standard orthogonal array L_{32} (2^{31}) was employed to construct the consortia. Six fungi were chosen as the six factors, and the influence of two-way interactions were also evaluated. The spore concentrations of each fungus in 1 mL of working spore suspensions were selected as levels. Each factor was assigned 0 spore/mL of spore concentration as the low level and 1×10^7 spores/mL as the high level. Subsequently, the data were subjected to analysis of variance (ANOVA) to evaluate the main and interaction effects of different fungi on VR degradation.

Optimization of Culture Conditions

Various process parameters influencing the reducing sugar release and enzyme activity during SSF were identified. These included initial pH, moisture content, temperature, harvest time, inoculum size, urea content, and $MnSO_4$ content. Moisture content was adjusted using various volumes of mineral medium, and initial pH was adjusted with 5 M NaOH or 1 M HCl (Latifian *et al*. 2007).

Determination of Fiber

Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and ash were determined using filter bags (ANKOM) as described by Van Soest *et al.* (1991). The fiber fractions were calculated as follows: hemicellulose = NDF — ADF, cellulose = ADF — ADL, lignin = ADL — ash (El-Zoghbi 1994).

Fungal Populations Analysis

The amount of fungi was determined by the dilution method of plate counting. Plate counts were performed by the spread plate method. The counting media were solidified using 18 g per liter of agar powder. After thorough dispersion, the sample was diluted serially and plated in duplicate to obtain the viable count.

Statistical Analysis

All fermentation experiments were carried out in triplicate. Statistical analysis was performed using SAS version 9.2. The data were analyzed using ANOVA, and significant means were tested with Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Fungal Consortia Construction for VR Degradation using Orthogonal Design

VR consists primarily of rice chaff (Fig. 1), which cannot be degraded as much as straw. The lack of nutrients does not make VR a good natural culture medium, which requires the degraders to survive in this medium and then initiate the process of degradation. In the present work, the viable counts of *P. chrysosporium*, *T. koningii*, *Trichoderma viride*, *A. niger*, *A. ficuum* NTG-23, and *Candida utilis* increased over time (Fig. 2) in their individual pure cultures. These results show that all the selected fungi could grow well in this natural culture medium.

Fig. 1. The raw vinegar residue on an air-dry basis

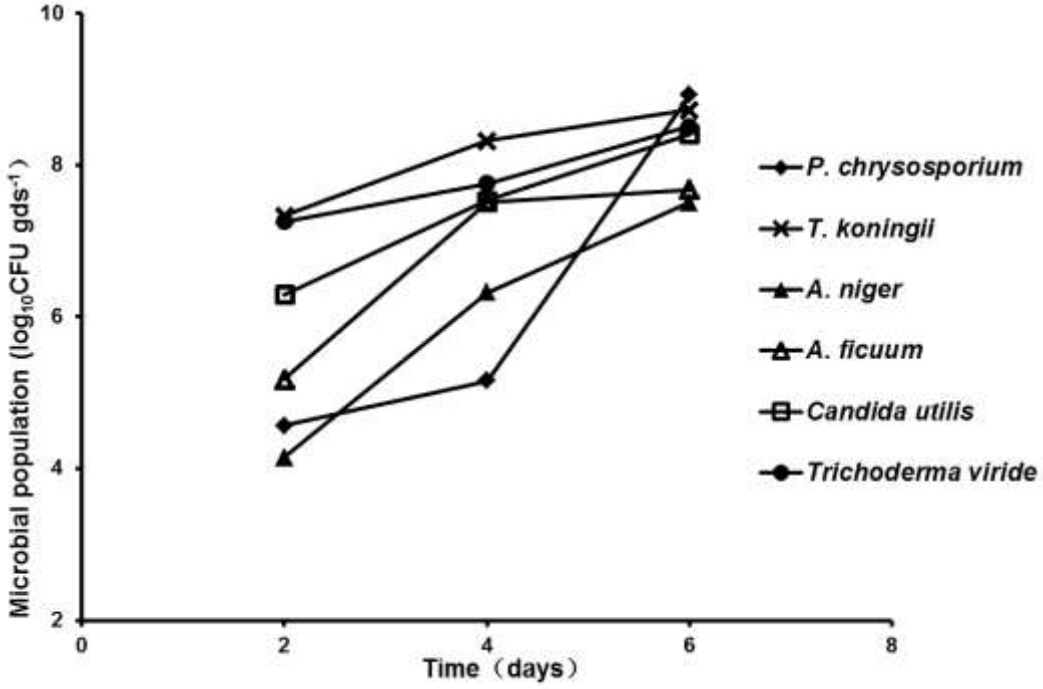


Fig. 2. Data (mean) were calculated from three replicates on a dry matter (DM) basis; the results are expressed as log_{10}CFU

SSF of lignocellulosic wastes has several advantages over submerged fermentation (SmF), such as decreased operational costs, low capital costs for equipment, and high volumetric productivity (Villas-Bôas *et al.* 2002; Yang *et al.* 2004). Hence, it was used in this study. Reducing sugar primarily contains glucose, fructose, galactose, lactose, and maltose, all of which are monosaccharides or disaccharides and very easily digested. Hence, microbial degradation of highly ordered carbohydrate polymers into reducing sugar not only intenerates VR, increasing the palatability, but also enhances the content of digestible carbohydrates and hydrolytic enzymes, which might make the product a potential renewable feedstuff and avoid environmental pollution.

Table 1. Orthogonal Design of Fungal Consortia for Raw VR Degradation

No.	A	B	C	D	E	F	Reducing sugar (mg gds^{-1})
1	1	1	1	1	1	1	17.10±0.45
2	1	1	1	1	2	2	22.98±1.02
3	1	1	2	2	1	1	18.05±0.04
4	1	1	2	2	2	2	17.64±0.14
5	1	2	1	2	1	1	25.63±2.44
6	1	2	1	2	2	2	19.73±1.14
7	1	2	2	1	1	1	14.42±1.24
8	1	2	2	1	2	2	16.43±0.68
9	1	1	1	2	1	2	21.49±1.53
10	1	1	1	2	2	1	19.13±1.75
11	1	1	2	1	1	2	19.05±1.54
12	1	1	2	1	2	1	16.60±1.54
13	1	2	1	1	1	2	18.26±0.04
14	1	2	1	1	2	1	14.83±0.16
15	1	2	2	2	1	2	22.28±1.06
16	1	2	2	2	2	1	17.60±0.04
17	2	1	1	2	1	1	21.34±0.71
18	2	1	1	2	2	2	22.19±2.11
19	2	1	2	1	1	1	20.66±0.76
20	2	1	2	1	2	2	13.35±0.31
21	2	2	1	1	1	1	24.58±0.59
22	2	2	1	1	2	2	28.26±2.38
23	2	2	2	2	1	1	22.94±2.34
24	2	2	2	2	2	2	21.63±0.07
25	2	1	1	1	1	2	24.25±0.05
26	2	1	1	1	2	1	19.15±0.97
27	2	1	2	2	1	2	30.13±0.08
28	2	1	2	2	2	1	21.89±1.26
29	2	2	1	2	1	2	35.57±0.78
30	2	2	1	2	2	1	23.10±1.27
31	2	2	2	1	1	2	26.89±0.04
32	2	2	2	1	2	1	21.58±1.29

* A: *Phanerochaete chrysosporium,* B: *Trichoderma koningii,* C: *Trichoderma viride,* D: *Aspergillus niger,* E: *Candida utilis,* F: *Aspergillus ficuum* NTG-23, 1: spore concentration = 0 spore/mL, 2: spore concentration = 1×10^7 spores/mL.

Among the 32 consortia constructed using the orthogonal design (Table 1), consortium 29, consisting of *P. chrysosporium*, *T. koningii*, *A. niger*, and *A. ficuum* NTG-23, demonstrated the strongest VR decomposition ability, with a reducing sugar yield of 35.57 mg gds^{-1}. Analysis of variance results in Table 2 show that all of the six strains significantly ($p < 0.01$) affected the VR decomposition ability of the fungal consortia. Interaction impact investigation of strains × strains on the VR decomposition was carried out to construct an effective degradation fungal consortium. According to an estimation of margin means in Table 2, the interactions of *Trichoderma viride* × *P. chrysosporium*, *Trichoderma viride* × *T. koningii*, *Trichoderma viride* × *A. niger*, *Trichoderma viride* × *Candida utilis*, and *Trichoderma viride* × *A. ficuum* NTG-23 on the VR degradation were negative. Similarly, the interactions of *Candida utilis* × *P. chrysosporium*, *Candida utilis* × *T. koningii*, *Candida utilis* × *A. niger*, *Candida utilis* × *Trichoderma viride*, and

Candida utilis × *A. ficuum* NTG-23 on the VR degradation were also negative. The mixed cultivation of *Trichoderma viride* or *Candida utilis* with any of the other five fungi presented an inhibitory effect on the VR degradation. These results suggested that *Trichoderma viride* and *Candida utilis* might not cooperate well with the other five strains. Conversely, the interaction effects of *P. chrysosporium* × *T. koningii*, *P. chrysosporium* × *A. niger*, *P. chrysosporium* × *A. ficuum* NTG-23, *T. koningii* × *A. niger*, *T. koningii* × *A. ficuum* NTG-23, and *A. niger* × *A. ficuum* NTG-23 were found to be positive. These results indicated that *P. chrysosporium*, *T. koningii*, *A. niger*, and *A. ficuum* NTG-23 might act synergistically in a consortium; thus, the fungal consortium-29 was thought to be the optimal fungal consortium for VR degradation.

Table 2. ANOVA and Estimation of the Two-way Interactions between Strains

Factor	Margin Means	P-value	Factor	Margin Means	P-value
a	a1＜a2	＜0.0001	b∗c	b2c2＜b2c1	0.0772
b	b1＜b2	0.0018	b∗d	b2d1 (or b1d2)＜b2d2	0.6115
c	c2＜c1	0.0001	b∗e	b2e2＜b2e1	0.3563
d	d1＜d2	＜0.0001	b∗f	b2f1 (or b2f2)＜b2f2	0.4194
e	e2＜e1	＜0.0001	c∗d	c2d2＜c1d2	0.6204
f	f1＜f2	＜0.0001	c∗e	c2e2＜c2e1	0.3222
a∗b	a2b1 (or a1b2)＜a2b2	0.0002	c∗f	c2f2＜c1f2	0.1142
a∗c	a2c2＜a2c1	0.7978	d∗e	d2e2＜d2e1	0.0134
a∗d	a2d1 (or a1d2)＜a2d2	0.8390	d∗f	d2f1 (or d1f2)＜d2f2	0.9621
a∗e	a2e2＜a2e1	0.0090	e∗f	e2f2＜f1g2	0.0065
a∗f	a2f1 (or a1f2)＜a2f2	0.1623			

* a: *Phanerochaete chrysosporium*, b: *Trichoderma koningii*, c: *Trichoderma viride*, d: *Aspergillus niger*, e: *Candida utilis*, f: *Aspergillus ficuum* NTG-23, 1: spore concentration = 0 spore/mL, 2: spore concentration = 1×10^7 spores/mL.

Evaluation of the Lignocellulose Degradation Ability of Consortium-29

The reducing sugar release in consortium-29 was prominently higher than that of the pure *P. chrysosporium* culture, which presented the highest reducing sugar yield among all the single fungus pure culture (Fig. 3a). In fact, the content of reducing sugar in the product increased by 108.01% as compared with the control. In previous reports, mixed fermentation of *Trichoderma* sp. and *Aspergillus* sp. has proven to be an excellent candidate for the production of cellulolytic enzymes with strong hydrolytic ability because of the complementary interactions of cellulases from *Trichoderma* sp. strains and *Aspergillus sp*. strains. *Trichoderma* strains can secrete both endo- and exo-glucanase with high activities, with very low β-glucosidases activity, while strains of *Aspergillus* show high activity of β-glucosidases (Brijwani *et al*. 2010). *P. chrysosporium* degrades lignin efficiently and selectively using its ligninolytic enzyme system, which primarily comprises lignin peroxidase, manganese peroxidase, and laccase (Arora *et al*. 2002).

In this study, remarkably stronger degradation capacities were displayed by *P. chrysosporium*, *T. koningii*, and *A. ficuum* NTG-23 compared with the other three fungi (Fig 3a), indicating the important roles of these three strains in effective degradation fungal consortia. *A. niger* presented notably higher xylanase activity (Fig 3b) than the other five strains, which was similar to previous reports (Pal and Khanum 2010). Therefore, consortium-29, consisting of *P. chrysosporium*, *T. koningii*, *A. niger*, and *A.*

ficuum NTG-23, showed strong degradation ability because of its lignocellulolytic enzyme system, with an appropriate composition and good catalyzing characteristics, which was in accordance with the co-cultivation of specific fungi (Fang *et al.* 2010). Additionally, the xylanase and CMCase activity reached 439.07 U gds^{-1} and 8.15 U gds^{-1}, which were 432.08% and 243.88% higher than that of the pure culture of *A. niger* (82.52 U gds^{-1}) and *P. chrysosporium* (2.37 U gds^{-1}), respectively. These findings also indicate the strong degradation capacity of consortium-29.

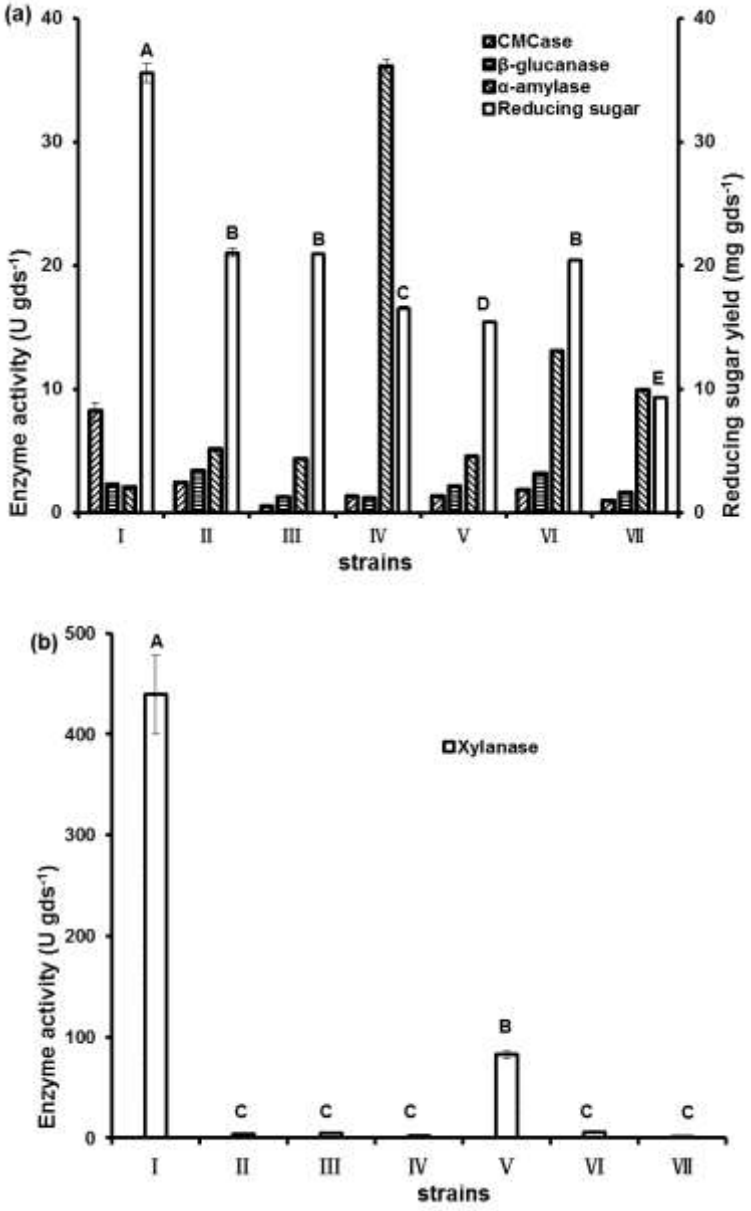


Fig. 3. Evaluation of lignocellulolytic enzyme activities and reducing sugar production of consortium-29 and individual pure cultures (spore concentration of the inoculum = 1×10^7 spores/mL). Error bars represent the standard deviation (n = 3). $^{A\text{-}E}$ Means values with different letters differ significantly ($p < 0.01$). The strains of I, II, III, IV, V, VI, VII were Consortium-29, *P. chrysosporium*, *T. koningii*, *Trichoderma viride*, *A. niger*, *A. ficuum* NTG-23, and *Candida utilis*, respectively. (a) Reducing sugar yield and enzyme activity. (b) Xylanase activity.

The content of fiber components and ash in fermented products obtained by various fermentation strains is presented in Fig. 4. According to the analysis of fiber fractions, all the contents of NDF, ADF, ADL, cellulose, hemicellulose, and lignin in the products of consortium-29 were the lowest among all the treatments (Fig. 4a-f). These results show that consortium-29 had significantly stronger lignocellulose (cellulose, hemicellulose, and lignin) decomposition ability than any of the six pure strains. In fact, through the treatment of consortium-29, the contents of cellulose, hemicellulose, and lignin decreased by 17.11%, 68.61%, 14.44% respectively, as compared with that of the raw VR. The ratio of NDF degradation reached 22.03%, which was equal to that of mixed culture solid fermentation of NaOH pretreated rice chaff by *Trichoderma reesei*, *Aspergillus niger*, and *Saccharomyces cerevisiae* under the optimal conditions (Yong *et al.* 2004). This result showed the high efficiency of consortium-29. Moreover, the percentage of ash in the fermented products of consortium-29 was the highest (Fig. 4g), while that in the raw VR was the lowest. These findings imply that the dry matter losses during fermentation by consortium-29 were the highest. In this case, the content of lignocellulose (hemicellulose, cellulose, and lignin) was still the lowest (Fig. 4d-f), which further demonstrated the strong lignocellulose degradation ability of consortium-29.

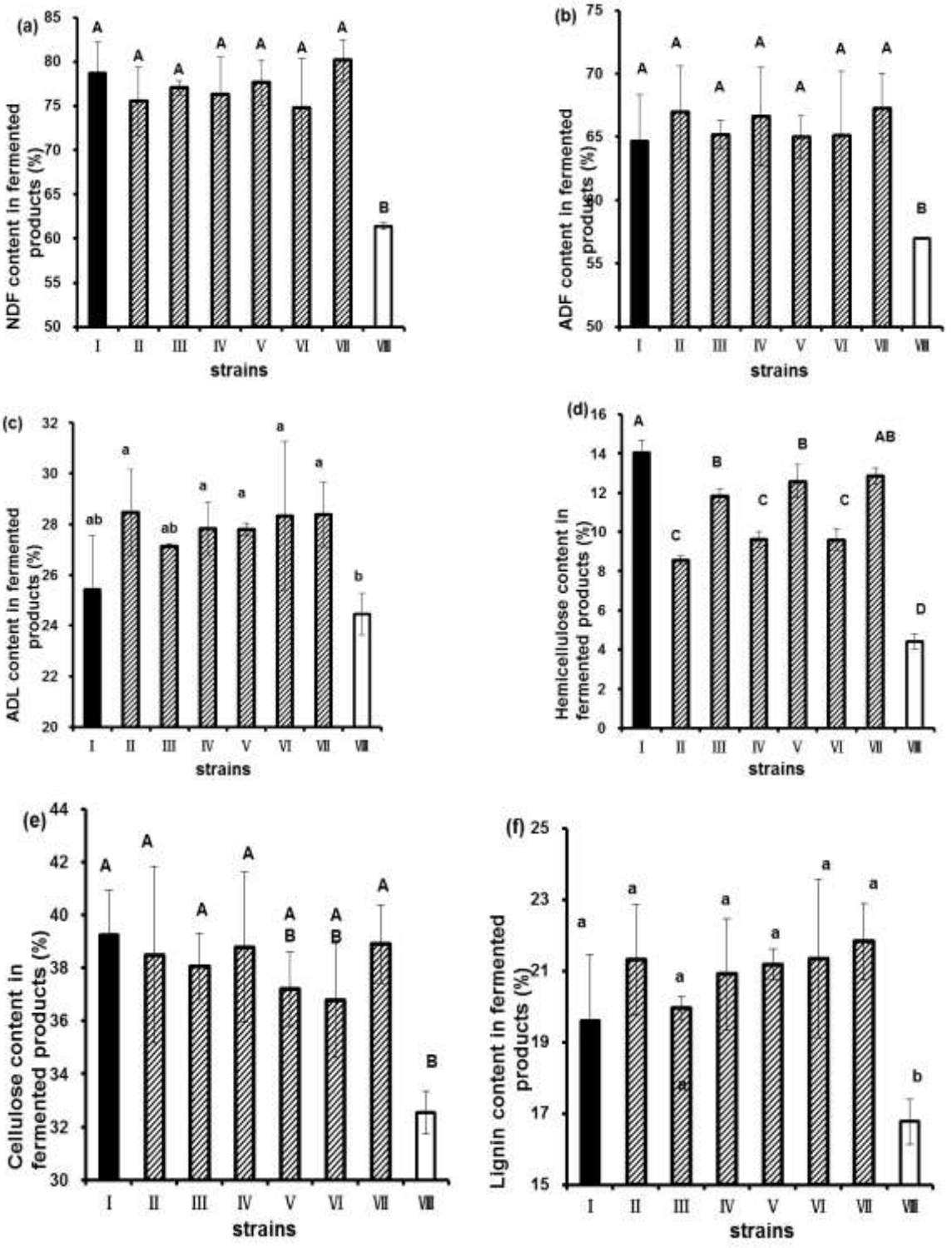
Optimization of the Culture Conditions of Consortium-29 for VR Degradation

Determining optimal conditions

The culture conditions were optimized according to an orthogonal experiment (Tables 3 and 4). From the value of R (Table 5), it was implied that the effects of the factors on reducing sugar yield in order of importance were incubation temperature, incubation time, urea content, $MnSO_4 \cdot H_2O$ content, inoculum size, initial pH, and initial moisture content. The optimal conditions were an incubation temperature of 25 °C, initial pH of 6， inoculum size 1×10^6 or 1×10^7 spores/mL, incubation time 5 days, urea/VR 1% (w/w), and $MnSO_4 \cdot H_2O$/VR 0.03% (w/w).

According to the statistical analysis (Table 5), it was found that incubation temperature, inoculum size, incubation time, urea content, and $MnSO_4 \cdot H_2O$ content had very significant effects on the release of reducing sugars ($p < 0.01$); initial pH had a significant effect on this release ($0.01 < p < 0.05$); and initial moisture content had no significant effect ($p > 0.05$).

The values of CMCase activity were analyzed to obtain the R value (Table 5), which indicated that the effects of the factors on CMCase activity in order of importance were urea content, incubation time, $MnSO_4 \cdot H_2O$ content, incubation temperature, initial pH, inoculum size, and initial moisture content. The optimal conditions were as follows: incubation temperature of 30 or 35 °C, initial pH of 5 or 6, initial moisture content of 70% (w/w), inoculum size of 1×10^6 spores/mL, incubation time of 5 days, urea/VR 1% (w/w), and $MnSO_4 \cdot H_2O$/VR 0.03% or 0.06% (w/w).


(a)
NDF content in fermented products (%)
strains
(b)
ADF content in fermented products (%)
strains
(c)
ADL content in fermented products (%)
strains
(d)
Hemicellulose content in fermented products (%)
strains
(e)
Cellulose content in fermented products (%)
strains
(f)
Lignin content in fermented products (%)
strains
I II III IV V VI VII VIII

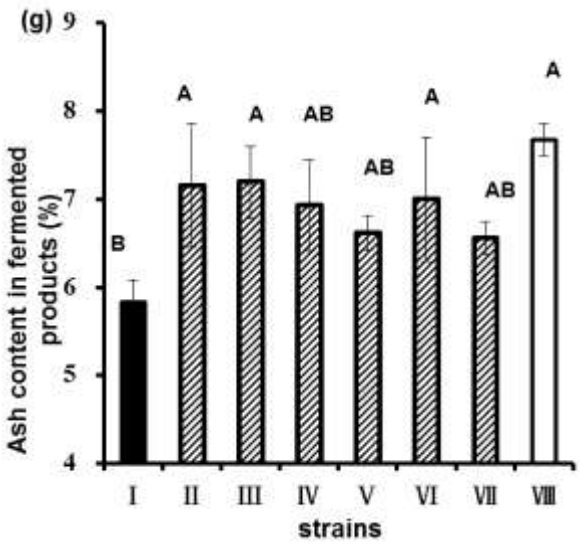


Fig. 4. Data (mean ± SD) for each treatment were calculated from three replicates on a dry matter (DM) basis. $^{A-D}$ Means values with different letters differ significantly ($p < 0.01$); $^{a-b}$ Means values with different letters differ significantly ($p < 0.05$). The strains of I, II, III, IV, V, VI, VII, VIII are non-strains, *P. chrysosporium*, *T. koningii*, *Trichoderma viride*, *A. niger*, *A. ficuum* NTG-23, *Candida utilis*, and consortium-29, respectively.

Table 3. Factors and their Levels for Orthogonal Experiments

Levels	Factors						
	G	H	I	J	K	L	M
1	25	5	50	1×10^6	5	1	0.03
2	30	6	60	1×10^7	10	2	0.06
3	35	7	70	1×10^8	15	3	0.12

* G: Incubation temperature (°C); H: Initial pH; I: Initial moisture content (w/w %); J: Inoculum size (spores/mL); K: Incubation time (days); L: Urea/VR (w/w %); M: $MnSO_4 \cdot H_2O$/VR (w/w %).

It was found that incubation temperature, initial pH, inoculum size, incubation time, urea content, and $MnSO_4 \cdot H_2O$ content had very significant effects on CMCase activity ($p < 0.01$); and initial moisture content had a significant effect on it ($0.01 < p < 0.05$).

Effect of factor level

Temperature was the key physical variable in the SSF. In this work, the optimal temperature for the reducing sugar release and CMCase activity in the fermented product of consortium-29 was investigated. The maximum reducing sugar yield occurred at 25 °C (Table 5), which is similar to a large amount of fungi having relatively low optimal temperature in their cultures (Kim *et al*. 2005). It is well known that fungi favor a moist environment as they grow. The optimal moisture content during the SSF depends on the natural requirements of the microbe, the type of the end products, and the nature of the substrate (Kalogeris *et al*. 2003a). Hence, the influence of moisture content was investigated for VR degradation by consortium-29.

Table 4. Analyses of the Reducing Sugar Yield and CMCase Activity in the Orthogonal Experiment

No.	Factors							Results	
	G	H	I	J	K	L	M	Reducing Sugar (mg gds^{-1})	CMCase (U/g)
1	1	1	1	1	1	1	1	41.67±2.60	9.35±0.64
2	1	1	1	1	2	2	2	23.31±1.10	9.40±0.35
3	1	1	1	1	3	3	3	21.39±0.65	3.08±0.09
4	1	2	2	2	1	1	1	38.46±1.04	9.52±0.01
5	1	2	2	2	2	2	2	27.08±0.84	6.79±0.06
6	1	2	2	2	3	3	3	23.39±0.97	3.06±0.24
7	1	3	3	3	1	1	1	34.76±0.48	10.46±0.06
8	1	3	3	3	2	2	2	20.97±0.75	4.77±0.40
9	1	3	3	3	3	3	3	21.83±1.73	3.18±0.26
10	2	1	2	3	1	2	3	22.48±1.65	8.04±0.20
11	2	1	2	3	2	3	1	18.64±1.34	5.53±0.27
12	2	1	2	3	3	1	2	19.19±1.37	9.62±0.39
13	2	2	3	1	1	2	3	24.92±0.05	8.88±0.41
14	2	2	3	1	2	3	1	19.62±0.27	7.41±0.11
15	2	2	3	1	3	1	2	21.28±1.01	9.37±0.37
16	2	3	1	2	1	2	3	19.88±1.66	6.07±0.38
17	2	3	1	2	2	3	1	20.06±0.61	5.47±0.31
18	2	3	1	2	3	1	2	23.59±1.20	9.48±0.72
19	3	1	3	2	1	3	2	18.98±0.30	7.72±0.06
20	3	1	3	2	2	1	3	20.50±0.73	9.98±0.70
21	3	1	3	2	3	2	1	17.26±0.04	7.74±0.05
22	3	2	1	3	1	3	2	18.98±0.72	7.03±0.13
23	3	2	1	3	2	1	3	21.63±2.69	8.19±0.28
24	3	2	1	3	3	2	1	16.65±0.75	6.65±0.02
25	3	3	2	1	1	3	2	20.83±1.36	7.66±0.39
26	3	3	2	1	2	1	3	20.40±1.91	8.22±0.23
27	3	3	2	1	3	2	1	17.58±0.75	6.83±0.27

The highest CMCase activity was obtained when the initial moisture content was 70% (Table 5), which might be due to the faster growth of fungi at high moisture content and the earlier initiation of enzyme production in the subsequent fermentation. Referring to previous reports, it was found that high moisture content enhanced the microbial growth and lignocellulolytic enzyme system production when lignocellulosic substrates were used as the carbon sources in the SSF (Kalogeris *et al*. 2003b).

The maximum reducing sugar production and the highest CMCase activity were acquired when the initial pH was 6 and 5 (Table 5), respectively. This property suggested that consortium-29 could be used to degrade lignocellulosic wastes and produce hydrolytic enzyme under weak acidic conditions.

An inoculum size that is too large leads to intense competition for oxygen and nutritive substances, which could decrease synthetic products. However, an inoculum size that is too small might result in longer incubation time and low fermentation productivity. Therefore, the optimal inoculum size was investigated in this study. The optimal reducing sugar production was obtained when the inoculum size was 1×10^7 or 1×10^6 spores/mL, and the optimal CMCase activity was exhibited when the inoculum size was 1×10^6 spores/mL.

The incubation time required to reach maximum levels of degradation might be affected by different ratios of amorphous to crystalline cellulose (Ögel *et al*. 2001). Therefore, the fermentation time was further analyzed. Both the highest reducing sugar release and CMCase activity were observed after five days (Table 5). These results are similar to previous reports (Yang *et al*. 2004; Feng *et al*. 2011). These findings showed that aerobic metabolism prevailed during the first five days, and that, in subsequent fermentation stages, oxygen was limited and the reducing sugar was converted to other products (Feng *et al*. 2011).

In previous reports, inorganic nitrogen sources were optimal for the acquisition of maximum lignocellulolytic enzyme activity (Kalogeris *et al*. 2003a). Urea, the most important inorganic nitrogen source, was used in this study. Both the maximum reducing sugar production and the highest CMCase activity were obtained when the content of urea was 1% (Table 5).

Manganese plays an important role in lignin biodegradation by white rot fungi, both as an active mediator for Mn peroxidase and as a regulator for lignin peroxidase, manganese peroxidase, and laccase secretion (Kerem and Hadar 1995). Therefore, the effect of Mn content was investigated. The maximum reducing sugar production and the highest CMCase activity were acquired when the $MnSO_4 \cdot H_2O$ contents were 0.03% and 0.06% (Table 5), respectively. The optimal Mn content of consortium-29 for CMCase activity was similar to the reported optimal $MnSO_4$ content (600 $\mu g g^{-1}$) for *Pleurotus ostreatus* (Kerem *et al*. 1995).

Taking both reducing sugar yield and CMCase activity into account, the optimal fermentation conditions for lignocellulosic component degradation were as follows: incubation temperature of 25 °C, initial pH of 6, initial moisture content of 70% (w/w), inoculum size of 1×10^6 spores/mL, incubation time of 5 days, urea/VR ratio 1% (w/w), and $MnSO_4 \cdot H_2O$/VR ratio 0.03% (w/w).

Table 5. Analyses of the Effect of Factors on the Reducing Sugar Yield and CMCase Activity

Parameters	G	H	I	J	K	L	M
Reducing Sugar							
k1	28.10^a	22.60^b	23.24	23.44^a	26.77^a	26.83^a	25.19^a
k2	21.30^b	23.56^a	23.12	23.47^a	21.58^b	21.13^b	21.58^b
k3	19.20^c	22.43^b	22.23	21.68^b	20.24^c	20.64^b	21.82^b
R	8.90	1.12	1.01	1.79	6.53	6.19	3.36
P-value	＜0.0001	0.0437	0.0757	0.0003	＜0.0001	＜0.0001	＜0.0001
CMCase							
k1	6.62^b	7.83^a	7.19^b	7.80^a	8.30^a	9.35^a	7.66^a
k2	7.76^a	7.43^a	7.25^b	7.31^b	7.30^b	7.24^b	7.98^a
k3	7.78^a	6.91^b	7.72^a	7.05^b	6.56^c	5.57^c	6.52^b
R	1.16	0.92	0.53	0.75	1.75	3.78	1.46
P-value	＜0.0001	0.0006	0.0416	0.0053	＜0.0001	＜0.0001	＜0.0001
* $^{a-b}$ Values with different letters differ significantly ($p < 0.05$).							

All the optimal levels of temperature, inoculum size, incubation time, urea content and $MnSO_4$ content were the lowest that were considered, which means the lowest cost. The optimal pH is easily controlled. The moisture content of fresh VR is about 70%, in accordance with the optimal moisture content, which implies that the moisture of VR do

no need to adjust before fermentation. Moreover, about 40 to 45% of total projected cost for hydrolysis of lignocellulosic biomass is attributed to the process of sugar release from lignocellulosic materials, including pretreatment, enzyme production and enzymatic hydrolysis (Wooley *et al*. 1999; Yang and Wyman 2008; Guo *et al*. 2010; Hui *et al*. 2013). Besides, the cost of SmF is more than 4 times of that of SSF. SSF was adopted in this technology and VR substrate could directly use without being milled and pretreated. All the above mention bring down the cost, and make this technology a cost-competitive strategy for large-scale practical utilization of lignocellulosic wastes.

CONCLUSIONS

1. Fungal consortium-29 was constructed using orthogonal design combined with two-way interaction analysis based on the reducing sugar yield. It is composed of *P. chrysosporium, T. koningii, A. niger,* and *A. ficuum* NTG-23 and is an efficient candidate for lignocellulosic component degradation in un-pretreated vinegar residue.
2. Degradation of lignocellulosic components in un-pretreated vinegar residue using fungal consortium-29 by SSF is more efficient than using the individual pure culture and represents a cost-competitive strategy for lignocellulosic waste utilization.
3. The optimal fermentation conditions of consortium-29 for un-pretreated VR degradation were as follows: incubation temperature of 25 °C, initial pH of 6, initial moisture content of 70%, inoculum size of 1×10^6 spores/mL, incubation time of 5 days, urea/VR ratio 1%, and $MnSO_4 \cdot H_2O$/VR ratio 0.03%.

ACKNOWLEDGMENTS

This study was financially supported by a Grant (2011BAD26B01-2) from the National Key Technology R&D Program for the 12th Five-year Plan of China and the Special Fund for the Innovative Team of the Chinese Academy of Agricultural Sciences (ASTIP-IAS08).

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Article submitted: January 7, 2015; Peer review completed: March 22, 2015; Revisions received and accepted: April 16, 2015; Published: April 22, 2015.
DOI: 10.15376/biores.10.2.3434-3450