Optimization of the Hydrolysis Condition of Pretreated Corn Stover using *Trichoderma viride* Broth based on Orthogonal Design and Principal Component Analysis

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A new strategy is described to optimize multiple closely related parameters that are involved in the degradation of lignocellulose. Exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase contained in the broth of *Trichoderma viride* 3.3711 cultures were used as enzyme solution. Corn stover (CS) pretreated by a combination of H_2O_2 and lignin peroxidase was used as raw feedstock. A comprehensive hydrolysis index (CHI) of three enzymatic activities was constructed by principal component analysis (PCA). Corn stover (CS) was pretreated with a combination of H_2O_2 and lignin peroxidase. The accuracy of the CHI was demonstrated by a quadratic regression using the CHI as an independent variable and the yield of the total reducing sugar (Y_{trs}) as a dependent variable. The results showed that the CHI was closely post-correlated with Y_{trs} and could be used to optimize the fermentation medium components for *T. viride* cultures due to a highly significant correlation between CHI and Y_{trs}. Based on the CHI at 96 h, an optimal medium contained 0.6% fructose, 0.6% xylose, 0.3% bean pulp, 0.15% yeast extract, 0.12% KH_2PO_4, 0.004% $CaCl_2$, 0.008% $FeSO_4$, 0.006% $ZnSO_4$, 0.012% glycine betaine, and 0.004% polyethylene glycol. The maximum actual Y_{trs} was very near to the theoretical Y_{trs}.

Keywords: Comprehensive hydrolysis index; Principal component analysis; Orthogonal design; Cellulase; Quadratic regression

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INTRODUCTION

As an agricultural waste product, lignocellulosic biomass is constructed mainly of cellulose, hemicellulose, and lignin. The lignocellulose can be utilized as a raw material to be converted into valuable chemicals, food products (Sasaki *et al*. 1998), and/or bio-energy (Lal 2005). In recent years, the conversion of lignocellulose into bio-energy has attracted a great deal of interest among researchers (Tye *et al*. 2016; Zhang *et al*. 2016). However, only a very small amount of lignocellulose is ultimately utilized, leading to a great quantity of lignocellulose being discarded, resulting in a waste of natural resources as well as generating environmental pollution. Lignin encases the hemicellulose and the compact crystalline structure of cellulose to prevent them from interacting with cellulase and hemicellulase (Shi *et al*. 2008; Bellido *et al*. 2014; Wang *et al*. 2015). The crystalline structure reduces the accessible surface area between cellulase and cellulose and between hemicellulase and hemicellulose. Therefore, the bottleneck in the process of using

lignocellulose as feedstock to produce bio-ethanol is degradation of the lignin and destruction of cellulose's crystalline structure by means of an economical, highly efficient, and environmentally friendly pretreatment to improve the enzymatic saccharification efficiency. The various pretreatment methods that have been developed can been divided into three types: physical, chemical, and biological. Among the pretreatment methods, using hydrogen peroxide (H_2O_2) for oxidative degradation is quite promising (Ramadoss and Muthukumar 2015; Zhang *et al*. 2015; Cao *et al*. 2016; Qing *et al*. 2016). The catalysts used in the H_2O_2 driven oxidative degradation of lignin include alkali (Cao *et al*. 2016), inorganic salt (Qing *et al*. 2016; Ramadoss and Muthukumar 2015), and an enzyme (Zhang *et al*. 2015). The advantages of an enzymatic-H_2O_2 oxidative degradation of lignin include cost-effectiveness, rapidity, convenience, safety, environmental friendliness, the lack of a need for specialized equipment, and a high yield of total reducing sugars (Y_{trs}). However, the hydrolysis solution produced by this method contains abundant lignin degradation products that may affect the cellulase-catalyzed saccharification of cellulose that is conducted in the following step.

In the previous study by Zhang *et al.* (2013), *Aspergillus oryzae* CGMCC5992 was isolated from the sludge of the Yudai River in Jiangsu University and was found to remove the chemical oxygen demand (COD) from vinasse. When gallic acid was used as a substrate, it was able to synthesize lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Guo *et al*. 2013). A proteomic analysis has shown that the strain secretes LiP, endo-1,4-β-D-glucanase, and proteinase in the presence of corn stover (CS) (Guan *et al*. 2015). Zyr (1956) suggested that these enzymes play key roles in the degradation of lignin and aromatic compounds. Furthermore, the present authors developed a method to degrade the lignin in the CS by combining solid fermentation with an enzymatic hydrolysis, and the data showed that *A. oryzae* can grow well on CS pretreated with 3% H_2O_2. This method has demonstrated a higher synthesis of MnP and LiP, a greater disintegration of lignin, a shortened treatment time (from 50 days to 10 days), and an increase in lignin degradation (from 57.8% to 80%) compared to solid-state fermentation (Zhang *et al*. 2013). However, the methods were limited in use because of the need for a large amount of cover area and a long fermentation cycle. Recently, a combination of liquid-state fermentation and enzymatic hydrolysis was used to pretreat the CS, after which the Y_{trs} reached a maximum of 46.3% after the pretreated CS was saccharified by a commercial cellulase (Zhang *et al*. 2015). In the present study, the authors aimed to maximize the Y_{trs} using *Trichoderma viride* 3.3711 broth containing cellulase instead of a commercial cellulase to directly saccharify the pretreated CS to reduce the cost of the cellulase extraction and to lay the foundation for industrial ethanol fermentation using CS as the raw material.

The application of cellulases in industrial processes is limited by their production costs, especially the costs of producing the medium, and the low yield obtained from these enzymes. Cellulases belong to a class of multienzyme systems that mainly consist of endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-1,4-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), which synergistically act to convert cellulose to glucose (Zhang and Zhang 2013). The enhancement of the synergistic action of this multienzyme system is the key to improving the saccharification rate of lignocellulosic biomass and reducing the production costs. Thus, low-cost bioprocesses able to produce cellulase have been extensively studied. However, this multienzyme system contains three types of enzymes that can hydrolyze lignocellulosic biomass through the individual or joint action of multiple enzymes. Thus, it is difficult to obtain the maximum synergistic action of the multienzyme systems through

commonly used optimization strategies, such as orthogonal design and response surface methodology.

As a well-established technique, principal component analysis (PCA) (Jolliffe 1986) has been widely used for data analysis and dimensionality reduction (Sun *et al*. 2006; Tao *et al*. 2009; Guan *et al*. 2011a,b, 2012a,b). PCA can be used to find a sequence of orthogonal factors that represent the directions of the largest variance. Such an approach is used in many applications, including machine learning (Xu *et al*. 2014), image processing (Tao *et al*. 2007), neurocomputing, engineering, and computer networks, especially for large datasets.

Several filamentous fungi can produce cellulases, and *T. viride* is one of the most extensively studied cellulolytic fungi; it is industrially used for enzyme production (Nathan *et al*. 2014). In the present work, the CS pretreated with a combination of H_2O_2 and LiP was used as feedstock to optimize hydrolysis reaction condition using *T. viride* cultivation medium contain cellulase production. A comprehensive hydrolysis index (CHI) was constructed using PCA with the data of three types of cellulase actions from an orthogonal design, and the *T. viride* cultivation medium used for cellulase production was further optimized by orthogonal design using CHI as an index. The authors' current study lays a foundation for ethanol fermentation using CS as a raw material and provides a new strategy for the optimization of fermentation conditions containing multiple-response values, such as cellulase.

EXPERIMENTAL

Materials

The CS was purchased from a local farm (Zhenjiang, China), dried to constant weight at 105 ºC, ground into a fine powder, and sieved through a 0.25-mm sieve. All of the chemicals used were of analytical or reagent grade (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China).

Strains and Culture Conditions

In the present study, the authors used *A. oryzae* CGMCC5992 that was previously isolated from the sludge of the Yudai River in Jiangsu University and had deposited in the China General Microbiological Culture Collection Center. The *T. viride* 3.3711 was purchased from the China General Microbiological Culture Collection Center (Beijing, China). The two strains were cultured on potato dextrose agar (PDA) slants at 28 °C for 4 days, and were stored at 4 °C and passaged every 7 to 9 weeks.

LiP Preparation

The LiP was prepared according the method described by Zhang *et al*. (2015) with minor modifications. A total of 1×10^6 *A. oryzae* CGMCC 5992 spores were aseptically inoculated into a 250-mL Erlenmeyer flask containing 100 mL of potato dextrose (PD) medium and then were incubated at 35 °C on a rotary incubator shaker (125 rpm) for 24 h to produce a mass of pelleted cells (Qiangle Laboratory Equipment Co., Ltd, Suzhou, China). This pelleted culture was used as a seed culture for liquid-state fermentation. Subsequently, 10 mL of the seed culture was aseptically inoculated into a 250-mL Erlenmeyer flask containing 100 mL of minimal medium (pH 6.8 to 7.0). The medium was

composed of (per L): 30 g of CS powder, 2.5 g of glycerol, 2.5 g of maltose, 15 g of yeast extract, 4.5 g of ammonium sulfate ($(NH_4)_2SO_4$), 0.4 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 0.4 g of copper sulfate ($CuSO_4 \cdot 5H_2O$), 1 g of manganese sulfate ($MnSO_4 \cdot H_2O$), 0.6 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.1 g of Vitamin B_{12} (VB_{12}), and 0.004 g of glycine. After inoculation, the flask was incubated at 35 °C on a rotary incubator shaker (125 rpm) for 72 h. The broth was centrifuged, and the supernatant was collected to determine the LiP activity. After adjusted to 500 U/L LiP, The supernatant was used to pretreatment of CS.

Oxidative Degradation of CS by H_2O_2 Using *A. oryzae* CGMCC 5992 Broth as a Catalyst

Briefly, 2000 g of CS was mixed with 25 L of deionized H_2O in a 50-L hydrolysis steel tank (Zhenjiang Geri biotechnological Co. Ltd, China), was pretreated at 110 °C for 10 min, and cooled to 35 °C. Subsequently, 7.5 L of the enzyme solution (the aforementioned LiP-containing supernatant) was added to the tank, and the mixture was stirred at 100 rpm and preheated to 35 °C. Then, 10 L of H_2O_2 (1.5%, V/V) was added to the mixture at a flow rate of 50 mL/min. After hydrolysis for 8 h, the mixture was filtered, and the filtered residue was dried to a constant weight and was used as the raw material to optimize the capacity of *T. viride* 3.3711 broth to hydrolyze the CS.

Orthogonal Design

A total of 3.37111×10^6 *T. viride* spores were aseptically inoculated into a 250-mL Erlenmeyer flask containing 100 mL of PD medium and then incubated at 30 °C in a rotary incubator shaker (150 rpm) for 48 h to produce a pellet. The fermentation medium was inoculated with 10% (v/v) of the liquid seed culture and was incubated at 30 °C and 150 rpm to produce the cellulase-containing broth.

According to the authors' preliminary test, a 5^{11} factorial design, a factorial arrangement with 11 factors at all five levels, was employed to optimize the 11 fermentation components in addition to the pretreatment of the CS residue. These 11 fermentation components included fructose (A), xylose (B), corn steep liquor (C), bean pulp (D), yeast extract (E), KH_2PO_4 (F), $CaCl_2$ (G), $FeSO_4$ (H), $ZnSO_4$ (I), glycine betaine (J), and polyethylene glycol (K). Because of the 11 design variables and five levels in the orthogonal design, a minimum orthogonal matrix method was selected as the L_{50} (5^{11}), as noted in Table 7. The logical next step was to determine the values of the factors that lead to the best possible response (Di *et al*. 2003; Xu *et al*. 2002). Table 7 lists detailed experimental conditions for each assay. Thus, the fermentation medium of each assay of the orthogonal designs contained 2% CS and the other optimized components in each assay (Table 1). All of the fermentation media were sterilized at 120 °C for 30 min. The broth from each assay was filtered, and the supernatant was subjected to multiple analyses, including the activity levels of carboxymethyl cellulase (CMCase), filter paper cellulase (FPase), and cellobiase, as well as the yield of Y_{trs}.

CHI construction using PCA

The degradation of cellulose in pretreated CS by exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase can occur by a single enzyme or by the interaction between two enzymes. In addition to their single action, the joint action between different enzymes or the same enzyme should be also considered when constructing a CHI using PCA.

Table 1. Application of L_{50} (5^{11}) Orthogonal Assays to Optimize the Hydrolysis Capability of *T. viride* 3.3711 in Broth (%)

Run	A*	B	C	D	E	F	G	H	I	J	K
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0.15	0.15	0.15	0.15	0.04	0.004	0.002	0.002	0.004	0.004
3	0	0.3	0.3	0.3	0.3	0.08	0.008	0.004	0.004	0.008	0.008
4	0	0.45	0.45	0.45	0.45	0.12	0.012	0.006	0.006	0.012	0.012
5	0	0.6	0.6	0.6	0.6	0.16	0.016	0.008	0.008	0.016	0.016
6	0.15	0	0.15	0.3	0.45	0.16	0	0.002	0.004	0.012	0.016
7	0.15	0.15	0.3	0.45	0.6	0	0.004	0.004	0.006	0.016	0
8	0.15	0.3	0.45	0.6	0	0.04	0.008	0.006	0.008	0	0.004
9	0.15	0.45	0.6	0	0.15	0.08	0.012	0.008	0	0.004	0.008
10	0.15	0.6	0	0.15	0.3	0.12	0.016	0	0.002	0.008	0.012
11	0.3	0	0.3	0.6	0.15	0.12	0.012	0	0.004	0.016	0.004
12	0.3	0.15	0.45	0	0.3	0.16	0.016	0.002	0.006	0	0.008
13	0.3	0.3	0.6	0.15	0.45	0	0	0.004	0.008	0.004	0.012
14	0.3	0.45	0	0.3	0.6	0.04	0.004	0.006	0	0.008	0.016
15	0.3	0.6	0.15	0.45	0	0.08	0.008	0.008	0.002	0.012	0
16	0..45	0	0.45	0.15	0.6	0.08	0.016	0.004	0	0.012	0.004
17	0..45	0.15	0.6	0.3	0	0.12	0	0.006	0.002	0.016	0.008
18	0..45	0.3	0	0.45	0.15	0.16	0.004	0.008	0.004	0	0.012
19	0..45	0.45	0.15	0.6	0.3	0	0.008	0	0.006	0.004	0.016
20	0..45	0.6	0.3	0	0.45	0.04	0.012	0.002	0.008	0.008	0
21	0.6	0	0.6	0.45	0.3	0.04	0.012	0.004	0.002	0	0.016
22	0.6	0.15	0	0.6	0.45	0.08	0.016	0.006	0.004	0.004	0
23	0.6	0.3	0.15	0	0.6	0.12	0	0.008	0.006	0.008	0.004
24	0.6	0.45	0.3	0.15	0	0.16	0.004	0	0.008	0.012	0.008
25	0.6	0.6	0.45	0.3	0.15	0	0.008	0.002	0	0.016	0.012
26	0	0	0	0.45	0.6	0.12	0.008	0.002	0.008	0.004	0.008
27	0	0.15	0.15	0.6	0	0.16	0.012	0.004	0	0.008	0.012
28	0	0.3	0.3	0	0.15	0	0.016	0.006	0.002	0.012	0.016
29	0	0.45	0.45	0.15	0.3	0.04	0	0.008	0.004	0.016	0
30	0	0.6	0.6	0.3	0.45	0.08	0.004	0	0.006	0	0.004
31	0.15	0	0.15	0	0.3	0.08	0.004	0.006	0.008	0.016	0.012
32	0.15	0.15	0.3	0.15	0.45	0.12	0.008	0.008	0	0	0.016
33	0.15	0.3	0.45	0.3	0.6	0.16	0.012	0	0.002	0.004	0
34	0.15	0.45	0.6	0.45	0	0	0.016	0.002	0.004	0.008	0.004
35	0.15	0.6	0	0.6	0.15	0.04	0	0.004	0.006	0.012	0.008
36	0.3	0	0.3	0.3	0	0.04	0.016	0.008	0.006	0.004	0.012
37	0.3	0.15	0.45	0.45	0.15	0.08	0	0	0.008	0.008	0.016
38	0.3	0.3	0.6	0.6	0.3	0.12	0.004	0.002	0	0.012	0
39	0.3	0.45	0	0	0.45	0.16	0.008	0.004	0.002	0.016	0.004
40	0.3	0.6	0.15	0.15	0.6	0	0.012	0.006	0.004	0	0.008
41	0.45	0	0.45	0.6	0.45	0	0.004	0.008	0.002	0.008	0.008
42	0.45	0.15	0.6	0	0.6	0.04	0.008	0	0.004	0.012	0.012
43	0.45	0.3	0	0.15	0	0.08	0.012	0.002	0.006	0.016	0.016
44	0.45	0.45	0.15	0.3	0.15	0.12	0.016	0.004	0.008	0	0
45	0.45	0.6	0.3	0.45	0.3	0.16	0	0.006	0	0.004	0.004
46	0.6	0	0.6	0.15	0.15	0.16	0.008	0.006	0.006	0.008	0
47	0.6	0.15	0	0.3	0.3	0	0.012	0.008	0.008	0.012	0.004
48	0.6	0.3	0.15	0.45	0.45	0.04	0.016	0	0	0.016	0.008
49	0.6	0.45	0.3	0.6	0.6	0.08	0	0.002	0.002	0	0.012
50	0.6	0.6	0.45	0	0	0.12	0.004	0.004	0.004	0.004	0.016

*****Note: A: fructose; B: xylose; C: corn steep liquor; D: bean pulp; E: yeast extract; F: KH_2PO_4; G: $CaCl_2$; H: $FeSO_4$; I: $ZnSO_4$; J: glycine betaine; and K: polyethylene glycol

The CHI should include F_i, $F_{i,j}$, and $F_{i,i}$. The F_i (namely F_1, F_2, and F_3) respectively denoting endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase activities denotes their single activity on cellulose. The action of $F_{i,j}$ (namely $F_{1,2}$, $F_{1,3}$, and $F_{2,3}$) is equal to $C_i \times C_j$ and denotes the interaction of different enzyme molecules. In addition, $F_{i,i}$ is equal to $C_i \times C_i$ and denotes the interaction of the same enzyme molecules. Therefore, the authors performed a PCA with nine selected factors, including F_i, $F_{i,j}$, and $F_{i,i}$. Because β-glucosidase activities at 24 h were too low, and the activity of all the enzymes at 120 h were less than those at 96 h, all F_i, $F_{i,j}$, and $F_{i,i}$ data obtained from each assay of the orthogonal design at 48 h, 72 h, 96 h, and 120 h were used to construct the CHI.

Determination of Y_{trs} from the Saccharification of Pretreated CS

Briefly, 1 mL of the aforementioned the cellulase-containing broth of *T. viride*, 40 mL of 0.2 M acetate buffer solution (pH 4.8), and 2 g of the pretreated CS were mixed into 250-mL Erlenmeyer flask. After being fully mixed, the initial concentration of the reducing sugar before reaction was determined and designated as C_0. The saccharification reaction was conducted at 50 °C in a shaking bath (Changzhou Huaguan Instrument Manufacturing Co. Ltd, China) (120 rpm) for 120 h. A 1 mL sample was collected at 24 h, 48 h, 72 h, 96 h, and 120 h and immediately cooled in an ice bath to terminate the reaction. The reducing sugar concentration in each flask was designated as C_1. The Y_{trs} was calculated according to Eq. 1:

$$Y_{trs}(\%) = \frac{(C_1 - C_0) \times V}{G} \times 100 \quad (1)$$

where V is the volume of the reaction solution (mL), G is the weight of the total dry substrate (g), and C_1 and C_0 are the reducing sugar concentrations (g/mL).

Methods

Different cellulolytic enzymes, such as exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase, were quantified using the standard methods described by Ghose (1987), respectively. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze the substrate to 1 μmol of glucose in 1 min under the test conditions. LiP activity was determined according to Tien and Kirk (1984) on the ultraviolet and visible spectrophotometer (Shanghai Science Instrument Co. Ltd, China). The total lignin content (acid-soluble plus acid-insoluble lignin), cellulose and hemi-cellulose of the substrates and raw materials was determined as described by Sluiter *et al*. (2008). The reducing sugar concentration in the hydrolysis solution was determined according to the 3,5-dinitrosalicylic acid method (Miller 1959).

All of the tests were repeated at least three times, and the results are presented as the means due to their negligible standard errors. All data was fitted using Microsoft Excel and SPSS 17.0 (IBM, Ammonst City, USA) and Matlab software 7.0 (V2014b) (MathWorks, Natick, USA). The PCA analysis was performed using SPSS 17.0 software.

RESULTS AND DISCUSSION

The main components (dry weight basis) of the ground CS consisted of 26.2% hemicellulose, 32.1% cellulose, and 15.4% lignin. The lignin content of the pretreated CS by combination H_2O_2 and the broth *A. oryzae* CGMCC5992 containing 500 U/L LiP was 9.8%.

The activities of exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase of each assay at 48 h, 72 h, 96 h, and 120 h in the orthogonal designs is shown in Table 2. These three enzymes not only were involved in the saccharification of pretreated CS, but also increased the synergetic action of these three enzymes to improve the efficiency of cellulose saccharification using *T. viride* broth as catalyst. However, the conventional strategy for optimizing fermentation conditions is based on single indexes. Accordingly, CHI construction for the synergetic action of these three enzymes has become a crucial step to enhance the cellulose-degrading ability of *T. viride* broth.

Table 2. The Results of Orthogonal Designs and Data of Factors Affecting CHI

Run	Y_{trs}	F_1	F_2	F_3	Run	Y_{trs}	F_1	F_2	F_3
48 h					96 h				
1	9.44	39.23	17.77	18.94	1	17.80	52.67	23.34	21.30
2	24.00	77.52	35.29	36.75	2	42.20	129.0	55.80	55.56
3	4.20	22.21	10.77	9.87	3	11.28	44.38	19.82	19.06
4	7.00	29.83	13.72	13.72	4	10.88	43.26	19.35	20.18
5	6.20	28.03	12.45	10.66	5	13.60	50.88	22.58	24.88
6	37.8	108.7	48.06	39.88	6	47.68	146.3	62.39	65.97
7	3.80	24.45	11.41	11.41	7	10.40	41.92	18.78	22.41
8	8.60	29.38	13.53	13.53	8	11.36	44.61	19.92	17.71
9	10.60	32.51	14.88	15.47	9	14.40	53.12	23.53	24.09
10	57.4	152.5	66.98	64.85	10	77.40	235.2	98.11	96.99
11	8.64	36.99	16.80	16.80	11	21.76	73.72	32.29	30.81
12	24.60	79.09	35.52	35.85	12	28.08	91.41	39.81	32.60
13	2.72	20.42	9.68	9.68	13	5.76	28.93	13.26	14.58
14	62.6	92.18	80.08	77.95	14	88.80	272.4	113.1	108.5
15	61.68	185.5	78.74	66.08	15	87.80	253.1	107.1	103.5
16	14.88	54.46	24.65	24.65	16	26.40	83.57	36.48	41.11
17	8.40	25.57	11.89	11.89	17	11.28	44.38	19.82	18.61
18	65.00	189.5	81.20	77.95	18	84.60	268.3	96.09	94.63
19	27.60	83.12	30.81	30.81	19	34.96	110.7	47.99	43.24
20	28.40	70.58	31.25	29.92	20	35.28	111.6	47.72	50.97
21	5.80	20.87	9.87	9.87	21	8.96	37.89	17.06	17.71
22	65.60	199.6	87.24	87.24	22	77.40	232.0	99.58	98.55
23	53.00	153.4	65.86	65.63	23	80.00	242.8	102.1	101.9
24	20.20	64.31	26.89	26.89	24	55.60	159.0	69.44	66.19
25	6.40	26.69	12.37	12.37	25	20.56	70.36	30.86	30.03
26	32.80	98.80	31.26	31.26	26	48.40	148.3	63.98	63.40
27	46.60	138.7	53.32	55.56	27	64.60	178.3	73.81	72.35
28	9.52	39.46	20.06	17.49	28	20.60	64.98	28.58	28.58
29	8.16	35.65	16.23	16.23	29	15.12	55.13	24.39	24.39
30	18.20	59.16	26.34	24.54	30	45.04	138.9	60.48	55.56
31	33.40	100.1	44.47	48.62	31	56.16	170.0	69.78	67.65
32	20.72	70.81	31.34	28.69	32	55.20	150.5	64.94	64.96
33	8.24	35.87	16.32	16.32	33	23.44	78.42	34.29	34.29

34	6.20	35.87	18.83	18.83	34	17.76	62.52	25.77	25.77
35	58.24	175.8	78.40	76.05	35	85.60	278.4	119.3	117.5
36	23.36	78.20	30.03	30.03	36	41.92	130.2	56.27	55.00
37	6.40	30.72	14.11	14.11	37	16.40	58.71	25.91	25.91
38	5.76	28.93	13.34	10.77	38	10.24	41.47	18.59	19.84
39	57.20	165.3	71.12	70.00	39	66.60	206.1	86.01	88.36
40	15.60	49.53	22.20	21.30	40	33.76	107.3	44.70	43.69
41	5.92	29.38	13.53	13.53	41	13.04	49.31	18.83	20.29
42	6.40	26.91	12.47	17.38	42	13.68	51.10	22.68	26.11
43	24.16	80.44	35.48	38.54	43	34.56	109.6	47.52	49.62
44	46.40	131.7	30.03	30.03	44	82.40	238.5	99.56	96.09
45	14.40	53.12	23.74	23.74	45	47.80	138.7	59.89	50.07
46	10.80	43.04	19.40	19.40	46	26.40	74.84	32.77	34.73
47	60.40	174.9	74.70	73.70	47	79.60	230.0	95.98	94.75
48	37.20	107.8	44.14	44.70	48	50.88	155.2	70.56	69.89
49	21.12	71393	31.82	31.15	49	40.20	130.8	54.89	52.53
50	6.56	31.17	14.30	14.58	50	36.40	111.3	42.91	48.73
72 h					120 h				
1	14.2	49.08	21.10	21.10	1	8.32	36.1	15.84	18.20
2	32.40	106.2	44.92	44.92	2	24.60	73.49	33.61	46.60
3	8.40	30.27	13.91	15.25	3	5.04	26.91	12.12	12.60
4	15.60	32.74	15.58	15.58	4	6.80	24.68	12.34	12.20
5	10.80	30.95	12.56	12.56	5	8.40	23.11	10.58	8.40
6	46.60	125.5	53.77	52.42	6	32.40	103.5	46.15	72.80
7	5.44	28.03	12.57	14.91	7	3.76	23.33	10.67	8.40
8	7.44	33.63	14.84	14.84	8	6.24	30.27	13.48	14.80
9	8.40	34.30	15.58	15.58	9	8.40	28.93	14.47	14.60
10	64.6	187.0	75.94	77.17	10	64.20	173.8	68.21	102.2
11	14.32	52.89	22.64	23.65	11	15.20	42.81	18.56	24.60
12	27.76	90.51	37.87	37.87	12	20.60	59.61	26.33	31.80
13	5.44	28.03	12.57	12.57	13	6.40	20.87	9.67	7.60
14	74.40	217.3	86.91	86.01	14	75.20	213.7	86.01	141.8
15	65.40	215.7	87.92	88.36	15	61.44	184.8	73.92	117.8
16	24.60	75.51	31.80	39.77	16	16.72	59.61	25.36	34.80
17	8.56	36.77	16.11	17.38	17	15.20	34.75	15.29	17.80
18	73.00	206.1	80.97	77.50	18	66.60	202.0	80.97	125.4
19	35.60	105.1	43.77	43.24	19	26.40	80.88	33.97	50.00
20	28.96	93.87	37.64	37.64	20	26.40	77.52	32.61	49.80
21	4.72	26.02	11.75	12.23	21	6.48	30.95	13.75	15.00
22	79.60	226.4	87.24	84.89	22	77.4	204.1	81.76	137.6
23	60.24	181.4	74.70	74.59	23	61.28	184.3	75.88	122.4
24	44.88	138.4	54.77	54.77	24	37.60	111.1	46.22	65.60
25	10.32	41.69	18.10	18.10	25	19.60	57.15	24.36	35.20
26	40.20	121.4	50.39	50.39	26	35.20	105.3	43.86	68.80
27	53.40	156.4	63.40	61.16	27	40.20	134.0	62.50	99.60
28	14.00	52.00	22.27	22.19	28	15.20	45.95	19.83	28.60
29	11.28	44.38	19.19	20.18	29	13.00	45.05	19.46	26.40
30	30.80	87.60	36.19	38.65	30	28.80	82.68	31.26	41.00
31	39.60	118.5	47.72	44.25	31	41.04	127.7	52.93	85.40
32	34.88	110.4	45.95	42.35	32	37.40	101.9	42.50	81.80
33	17.76	62.52	26.54	24.99	33	15.12	55.13	23.54	30.62
34	13.00	37.66	16.47	20.18	34	11.20	55.13	20.06	24.40
35	75.20	213.2	84.78	83.33	35	66.60	206.1	88.36	141.6
36	31.76	101.7	42.41	42.12	36	31.04	99.69	41.59	66.60

37	10.24	41.47	18.01	20.06	37	9.20	38.56	16.83	24.60
38	8.00	35.20	15.47	15.47	38	6.80	25.35	11.48	10.80
39	59.60	172.9	66.42	64.4	39	57.40	163.3	64.74	102.4
40	18.64	64.98	26.22	27.45	40	24.80	82.23	34.52	49.00
41	13.00	45.73	19.74	20.85	41	10.80	34.75	15.29	19.60
42	10.20	35.20	15.47	15.47	42	12.80	33.41	14.75	13.80
43	26.72	87.60	35.52	32.49	43	23.84	79.54	33.43	55.20
44	73.40	186.4	79.63	76.83	44	57.40	175.4	65.97	102.2
45	40.64	126.6	52.48	53.54	45	26.32	86.48	38.54	56.00
46	13.28	49.98	21.46	21.46	46	15.12	55.13	23.54	35.20
47	70.80	200.0	77.28	78.18	47	62.20	172.3	66.64	103.8
48	44.72	138.0	54.77	53.54	48	33.44	106.4	44.31	64.60
49	32.40	99.47	41.5	43.69	49	22.20	82.68	34.70	49.00
50	14.16	52.44	22.46	21.86	50	28.40	73.72	31.07	46.20

*Note: F_1: endo-β-1,4-glucanase; F_2: exo -β-1,4-glucanase; and F_3: β-glucosidase; the unit of Y_{trs} is %; the unit of F_1, F_2, F_3 is ($\times 10^{-2}$ U/mL)

Suitability test of CHI

The authors first analyzed whether these nine factors were suitable for PCA. Most of the correlation coefficients of the original variable matrix were close to 1, and most of the values were high. The p value of the correlation coefficient was 0.000, which indicated that it passed the significance test. In addition, there was a strong correlation among the original variables, so the use of a PCA was applicable.

Table 3. Kaiser-Meyer-Olkin Measure and Bartlett's Test of Sphericity

Kaiser-Meyer-Olkin Measure of Sample Adequacy		0.796
Bartlett's Test of Sphericity	Approximate Chi-square	9244.128
	Degrees of freedom	36
	Significance	0.000

Table 3 lists the results of the Kaiser-Meyer-Olkin (KMO) test and the Bartlett test. The KMO-value of the KMO test was 0.796, which showed significance, while the p-value of the Bartlett test was 0.000, less than an extremely significant level (0.01). These values also showed that the PCA was applicable to the raw data of nine factors.

Table 4. Initial Eigenvalues and Variance Contributions

Component	Initial Eigenvalues		
	Total	Variance (%)	Cumulative (%)
1	8.523	94.699	94.699
2	0.340	3.776	98.475
3	0.144	1.269	99.734
4	0.016	0.183	99.926
5	0.006	0.234	99.990
6	0.001	0.063	99.990
7	0.005	0.007	99.997
8	0.000	0.001	100.000
9	0.000	0.001	100.000

Factor Analysis and Evaluation

As an indicator of the CHI, the principal component (PC) is a linear combination of the components of the corresponding eigenvalues. Table 4 lists the total variance contributions of the extracted common factors after rotation and shows that the first PC could be screened because their total initial eigenvalues and variance contributions to CHI were greater than 1. The cumulative variance contribution rate of the PC was 94.699%, which included most of the information of nine factors.

To better display the significance of the PC, the extracted components were processed by conversion. Table 5 shows the loading matrix of the PC after the rotation of the maximum variance, indicating that polarization of the coefficient became more significant after rotation. The PC concentrated on all nine factors. The screened PC exhibited the information of nine factors in all directions.

Table 5 presents the score coefficient matrix of each factor of the PC by regression analysis. According to the coefficient of each item of the PC (Table 5), the component (PC) could be expressed by Eq. 2:

$$PC = 0.115F_1 + 0.115F_2 + 0.112F_3 + 0.115F_{1,1} + 0.115F_{2,2} + 0.108F_{3,3} + 0.115F_{1,2} + 0.116F_{1,3} + 0.116F_{2,3} \quad (2)$$

The corresponding variance contribution rate of the PC is shown in Table 3. The CHI could be expressed by Eq. 3:

$$CHI = \frac{94.699 \times PC}{94.699} = PC \quad (3)$$

Table 5. Loading Matrix and Score Coefficient Matrix of Each Item of the Principal Component after the Rotation

Item	Loading Matrix	Score Coefficient Matrix
F_1	0.977	0.115
F_2	0.977	0.115
F_3	0.956	0.112
F_1F_2	0.981	0.115
F_1F_3	0.992	0.116
F_2F_3	0.992	0.116
F_1F_1	0.979	0.115
F_2F_2	0.979	0.115
F_3F_3	0.922	0.108

After the PC was brought into Eq. 3, the relationship between the CHIs was expressed by Eq. 4 as follows:

$$CHI = 0.115F_1 + 0.115F_2 + 0.112F_3 + 0.115F_{1,1} + 0.115F_{2,2} + 0.108F_{3,3} + 0.115F_{1,2} + 0.116F_{1,3} + 0.116F_{2,3} \quad (4)$$

Table 6 shows the CHI of each run of the orthogonal design according to Eq. 4. Because the authors standardized the original data before analysis, the CHI presented here only showed the relative strong or weak hydrolysis capacity of the fermentation broth of each assay of orthogonal designs and could not show the actual hydrolysis capacity when the fermentation broth of each assay of orthogonal design was used as a crude enzyme liquor to hydrolyze the CS.

Table 6. The CHI of Each Run of the Orthogonal Design

Run	48 h	72 h	96 h	120 h	Run	48 h	72 h	96 h	120 h
1	0.1399	0.1806	0.1977	0.1258	26	0.4238	0.6874	0.9754	0.6563
2	0.3680	0.5622	0.7786	0.3804	27	0.8225	1.0125	1.2869	1.0762
3	0.0671	0.1012	0.1590	0.0847	28	0.1426	0.1952	0.2732	0.1875
4	0.0966	0.1113	0.1574	0.0795	29	0.1211	0.1602	0.2164	0.1783
5	0.0840	0.0945	0.1997	0.0659	30	0.2353	0.4193	0.8594	0.3895
6	0.5670	0.7342	0.9676	0.6757	31	0.5461	0.6335	1.1763	0.9065
7	0.0758	0.0926	0.1578	0.0665	32	0.3030	0.5770	1.0031	0.6939
8	0.0948	0.1102	0.1566	0.0994	33	0.1221	0.2483	0.3598	0.2314
9	0.1090	0.1150	0.2070	0.0979	34	0.1335	0.1357	0.2483	0.2047
10	1.0268	1.3944	2.0914	1.4043	35	1.3190	1.7028	2.8688	2.0960
11	0.1271	0.2027	0.3236	0.1651	36	0.3308	0.5193	0.7846	0.6083
12	0.3720	0.4352	0.4273	0.2576	37	0.1003	0.1491	0.2364	0.1493
13	0.0612	0.0880	0.0954	0.0584	38	0.0880	0.1167	0.1499	0.0766
14	1.4654	1.7702	2.6734	2.1490	39	1.1646	1.1591	1.6851	1.3067
15	1.3330	1.7800	2.3845	1.6320	40	0.1853	0.2628	0.5617	0.4282
16	0.2158	0.3583	0.4121	0.2637	41	0.0948	0.1669	0.1735	0.1243
17	0.0800	0.1262	0.1579	0.1203	42	0.0950	0.1167	0.2039	0.1073
18	1.4517	1.5792	2.3785	1.8800	43	0.3865	0.3953	0.6094	0.4363
19	0.3552	0.5452	0.5904	0.4243	44	0.5714	1.4105	2.1272	1.4021
20	0.3057	0.4482	0.6262	0.4047	45	0.2066	0.7395	0.8286	0.4889
21	0.0628	0.0813	0.1320	0.1019	46	0.1551	0.1851	0.3421	0.2446
22	1.6324	1.8448	2.0853	1.9969	47	1.2757	1.5105	2.0112	1.3931
23	1.0307	1.3288	2.2321	1.6742	48	0.5657	0.8160	1.0920	0.6449
24	0.2609	0.8245	1.0908	0.6824	49	0.3164	0.5113	0.7702	0.4308
25	0.0843	0.1453	0.3040	0.2537	50	0.1027	0.1961	0.5950	0.3711

Analysis of the interdependence of CHI and Y_{trs}

To clarify whether the constructed CHI was suitable to be used as an index to optimize the *T. viride* fermentation conditions for cellulase synthesis, the authors analyzed the interdependence between the CHI and the Y_{trs} of the pretreated CS hydrolyzed by *T. viride* broth in each assay of orthogonal designs (shown in the first column of Table 2). Many studies have reported that biomacromolecules, such as lignin, cellulose, and hemicelluloses, can adsorb enzymes and limit mass-transfer when using CS as a substrate (Rosgaard *et al*. 2007; Lee *et al*. 1994; Vallander and Eriksson 1987). When the enzyme concentration in the reaction solution was very low, the substrate (CS) adsorbed fewer enzymes, and the reaction velocity was proportional to the enzyme amount. However, the substrate adsorbed a larger number of enzymes when they were present in excess in solution. These adsorbed enzymes physically restrained the mass transfer and decreased the reaction velocities. Therefore, the interdependence between CHI and Y_{trs} was fitted by a simple and intuitive quadratic regression instead of linear regression as:

$$Y_{trs} = \alpha \times CHI^2 + \beta \times CHI + \gamma \quad (5)$$

where α and β are the coefficients of CHI^2 and CHI, respectively, and γ is a constant.

Table 7 shows the variance analysis of the relationship between the Y_{trs} and CHI. The F-value of the Fisher's test (4564.976) and p-value of the t-test (0.000) indicated that the correlation between Y_{trs} and CHI was extremely significant, and that the credibility of the analysis was extremely high. The fitness of the model was also confirmed by the regression correlation coefficient R^2 value (0.979), which was higher than 0.95, and equal

to the adjusted R^2 (0.979). The analysis demonstrated that a significant correlation existed between the Y_{trs} and CHI (Table 7). The scatter profile of the standard residuals from the regression equation was shown in Fig. 1. Most of the residuals were scattered in the middle score of CHI, and all residuals presented a good Gaussian distribution. According to Fig. 1, it was suggested that a significant correlation exists between the Y_{trs} and CHI.

Table 7. Coefficient of Regression Analyses of Each Item between Y_{trs} and CHI*

Item	Coefficient	Standard Error	*t*-value	Significance
Constant	0.034	0.007	5.121	0.000
CHI^2	-0.1	0.006	-17.433	0.000
CHI	0.586	0.015	37.891	0.000

*Note: F-value = 4564.976, significance p = 0.000, R^2 = 0.979, and adj-R^2 = 0.979

The coefficients for all parameters were extremely significant (p = 0.000). The negative coefficient of CHI^2 (Table 7) demonstrated that the curve of the Y_{trs} and CHI appeared to have an inverted-U-shaped relationship, with Y_{trs} having the maximum value. After the coefficient of each item in Table 7 was introduced into Eq. 6, the following equation was generated:

$$Y_{trs} = -0.1 \times CHI^2 + 0.586 \times CHI + 0.034 \quad (6)$$

The following equation was obtained by taking the derivation of Y_{trs} to CHI:

$$Y_{trs}' = -0.2 \times CHI + 0.586 \quad (7)$$

where Y_{trs}' is the derivative of Y_{trs}. If $Y_{trs}' = 0$, then CHI = 2.93. When CHI < 2.93, $Y_{trs}' > 0$, which could be explained by Y_{trs} being significantly positively correlated with CHI. Conversely, when CHI > 2.93, $Y_{trs}' < 0$, which could be explained by Y_{trs} being significantly negatively correlated with CHI. When CHI = 2.93, Y_{trs} reached a maximum value (89.25%). In Table 5, all CHI values were less than 2.93; thus Y_{trs} was significantly positively correlated with CHI. These results show that the CHI has some application values and could be used as an index to optimize the components of fermentation medium.

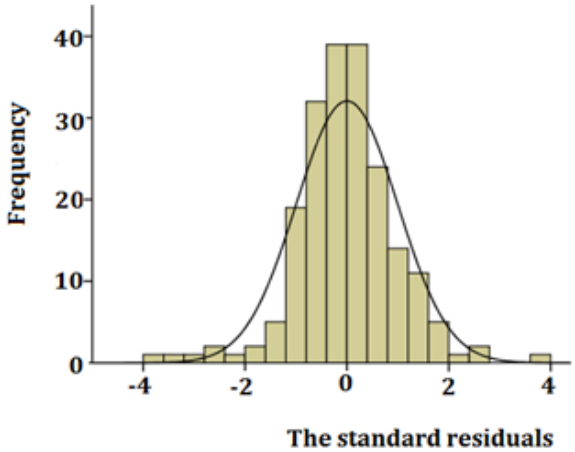

Fig. 1. The scatter profile of the standard residuals in the analysis of dependency between the yield of total reducing sugar and CHI

Optimization of Components by Orthogonal Design Using CHI as an Index

Based on a preliminary test, 11 fermentation components, including fructose (A), xylose (B), corn steep liquor (C), bean pulp (D), yeast extract (E), KH_2PO_4 (F), $CaCl_2$ (G), $FeSO_4$ (H), $ZnSO_4$ (I), glycine betaine (J), and polyethylene glycol (K), were screened and used as 11 independent variables, while CHI was used as a dependent variable (or response value). During the optimization, the fermentations were performed in a rotary incubator shaker at 150 rpm and 30 °C. The initial pH of the medium for all assays was adjusted to 6.0. Table 8 reveals that most CHIs of all the assays at 96 h were relatively higher; thus the authors used the CHIs at 96 h as the response values to optimize the components of the fermentation medium. The effects of all components in the culture medium on CHI were determined using range (R) analysis. The R analysis showed that the order of effects of all factors on CHI was C > B > H > A > F > J > G > D > E > K > I, namely, corn steep liquor > xylose > $FeSO_4$ > fructose > H_2PO_4 > glycine betaine > $CaCl_2$ > bean pulp > yeast extract > polyethylene glycol > $ZnSO_4$. Based on R-values, the optimum media components were $C_1B_5H_5A_5F_4J_4G_2D_3E_2K_2I_4$. Although the effect of the corn steep liquor was more important than the other nutrients, the CHI was the highest when *T. viride* 3.3711 was cultured in the medium without corn steep liquor. Therefore, the optimal fermentation medium consisted of 0.6% fructose, 0.6% xylose, 0.3% bean pulp, 0.15% yeast extract, 0.12% KH_2PO_4, 0.004% $CaCl_2$, 0.008% $FeSO_4$, 0.006% $ZnSO_4$, 0.012% glycine betaine, and 0.004% polyethylene glycol. Under those conditions, at 96 h in the process of fermentation, the activities of CMCase, FPase, and β-glucosidase were 2.835U/mL, 1.196U/mL and 1.175 U/mL and the maximum actual Y_{trs} was 90.5%. From the three enzymes activities, the CHI was calculated as 2.749 from equations (2) and (3) and the theoretical Y_{trs} from equation (6) was 88.92. The theoretical Y_{trs} value was very near to actual Y_{trs} value.

Table 8. Results of the Analysis of the Orthogonal Design

Run	A	B	C	D	E	F	G	H	I	J	K
k_1	0.510	0.548	1.758	0.762	0.751	0.461	0.857	0.705	0.815	0.861	0.865
k_2	0.924	0.835	1.320	0.720	0.984	0.953	1.003	0.586	0.881	0.730	0.954
k_3	0.932	0.751	0.602	1.040	0.778	0.890	0.780	0.952	0.774	1.007	0.771
k_4	0.811	0.977	0.304	0.859	0.875	0.981	0.628	0.841	0.903	1.052	0.925
k_5	1.065	1.132	0.260	0.860	0.855	0.957	0.974	1.159	0.870	0.592	0.728
R	0.555	0.584	1.498	0.320	0.233	0.520	0.375	0.573	0.129	0.460	0.226

CONCLUSIONS

1. A comprehensive hydrolysis index (CHI) containing single and multiple interactions among exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase was constructed *via* principal component analysis (PCA).
2. It was demonstrated that a quadratic regression model could fit the curve profile between the yield of the total reducing sugar (Y_{trs}) and CHI, and the CHI was significantly positively correlated with Y_{trs} under the test conditions.
3. The CHI analysis could reflect the capacity of the *T. viride* broth to hydrolyze corn stover (CS) pretreated by the *A. oryzae* fermentation broth.

4. The CHI was used as a response value to optimize the fermentation medium of *T. viride*. The optimal fermentation medium contained 0.6% fructose, 0.6% xylose, 0.3% bean pulp, 0.15% yeast extract, 0.12% KH_2PO_4, 0.004% $CaCl_2$, 0.008% $FeSO_4$, 0.006% $ZnSO_4$, 0.012% glycine betaine, and 0.004% polyethylene glycol.

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