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Full Length Article

Cellulase Production and Saccharification of Steam-exploded Corn Stover by Mutant Strain *Trichoderma reesei* EBUV-3

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Abstract

Numerous cellulase producing mutant strains were obtained from the parent strain *Trichoderma reesei* Rut C-30 treated with 10 μg/mL ethidium bromide (EtBr) for 20 min following by 30 min-UV irradiation. During strain screening, size of hydrolysis zone and FPase activity were served as the indexes and mutant *T. reesei* EBUV-3 was finally selected. Nearly 1.4, 1.7, 2.0 and 1.3 folds enhancement of the activities of FPase, CMCase, β-glucosidase and xylanase were noted in *T. reesei* EBUV-3 compared with *T. reesei* Rut C-30. The V_{max} of β-glucosidase and CMCase increased 1.7 and 1.4 folds. Meanwhile, the K_m values were 2.6 and 1.5 folds lower than the parent strain. The saccharification of steam-exploded corn stover by *T. reesei* EBUV-3 increased 24%. After 48 h saccharification, the reducing sugars production of *T. reesei* EBUV-3 was determined to be 70% at an optimal condition of 55°C and pH 5.0. Our present experimental findings suggested that mutant *T. reesei* EBUV-3 possesses a higher cellulase activity and stronger saccharifying ability compared with the parent strain. *T. reesei* EBUV-3 was promisingly potential to be a cellulase producer in application.

Keywords: Cellulase; Mutagenesis; *T. reesei* EBUV-3; Saccharification

Introduction

Cellulose is a water insoluble polymer composed of repeated units of β-D-glucopyranose interlinked by β-1, 4 glycosidic bonds (Sunkyu *et al*., 2010; Veeresh and Wu, 2014). It is the most abundant form of fixed carbon with 10^{11} tons produced in cell walls by plants each year (David, 2008). It can be exploited as a valuable and renewable carbon source for production energy. The key step in the utilization of cellulose is its hydrolysis into monomeric sugars with enzymatic hydrolysis or chemical hydrolysis, and subsequent conversion into valuable chemicals and energy (Veeresh and Wu, 2014). Thus, the concern about the production of glucose with plant biomass such as corn stover, fruit bunch and rice straw has increased (Marimuthu *et al*., 2014; SahaBadal and Cotta, 2014; Vandana and Anahit, 2014; Wang *et al.,* 2014; Xu *et al*., 2014). Enzymatic hydrolysis, which is an unpolluted way to convert cellulose to glucose, cost low comparing with chemical hydrolysis (Veeresh and Wu, 2014).

Cellulase is well acknowledged to use for saccharification of cellulose. Up to now, the cellulolytic fungus *Trichoderma reesei* Rut C-30 has been widely used for production of commercial cellulose (Vandana and Anahit, 2014). Cellulase is a multi-component enzyme comprising of endoglucanase (EC 3.2.1.74), which attack cellulose in amorphous zone and release oligomers, cellobiohydrolase (EC 3.2.1.91), that liberate cellobiose from reducing and non-reducing ends and β-glucosidase (EC 3.2.1.21), which hydrolyze cellobiose to glucose and play a key role in avoiding cellobiose inhibition and thus enhancing the hydrolysis rates of cellulose into glucose (David, 2008; Mehdi *et al*., 2010; Sunkyu *et al*., 2010; Baljit *et al.*, 2014; Veeresh and Wu, 2014).

In order to make those large-scale applications economically feasible, the cost of the cellulolytic enzymes needs to be reduced (David, 2008; Sunkyu *et al*., 2010; Veeresh and Wu, 2014). However, the cellulase productivity of this fungus could not satisfy the industry application. Thus, the production of cellulase must be increased. Recently, to enhance the cellulose production, strain mutation, genome reshuffling, protoplast fusion and recombinant protein expression technology have shown great potential (Mehdi *et al*., 2010; Baljit *et al*., 2014; Wang *et al*., 2014). Among these methods, strain mutation due to its efficiency and low-cost is still widely used to enhance the cellulase production.

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The aim of the present study was to obtain a higher activity strain for the hydrolysis and saccharification of corn stover.

Materials and Methods

Microorganisms and Medium

The strain of *Trichoderma reesei* Rut C-30 was obtained from the American Type Culture Collection (ATCC). The parent stain was maintained on potato dextrose agar (PDA) and sub-cultured once in every three months. The PDB (per liter) medium consisted of extract from 300 g of potatoes and 20 g of glucose, and the PDA medium contains 2% agar in PBD medium. Mandel's medium (per liter) was composed of 20 g KH_2PO_4, 3 g $CO(NH_2)_2$, 3 g $MgSO_4 \cdot 7H_2O$, 3 g $CaCl_2$, 0.05 g $FeSO_4 \cdot 7H_2O$, 0.0156 g $MnSO_4 \cdot H_2O$ and 0.014 g $ZnSO_4 \cdot 7H_2O$ (Simone and Studer, 2014). The mutants were maintained on the screening medium which contained 20% (v/v) swollen crystalline cellulose solution, 10% (v/v) Mandel's medium, 0.1% (w/v) peptone, 0.5% (w/v) 2-D-deoxyglucose and 2%(w/v) agar. The fermentation medium (per liter) supplemented with 30 g steam-exploded corn stover, 20 g bean flour, 3 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.3 g $CO(NH_2)_2$, 0. 3 g $MgSO_4$ and 5 g $CaCO_3$.

Corn Stover Pretreatments

The corn stover was obtained from a local company (Jilin Fuel Alcohol Company Ltd., China). The steam explosion pretreatment was performed in a 5.0 L vessel with the pressure of 1.50 MPa for 10 min and then dried at room temperature for enzymatic hydrolysis and component analysis.

Preparation of Mutants

The parent strain *T. reesei* Rut C-30 was grown on PDA slant for 14 days at 30°C. The conidia on slant were collected by adding phosphate buffer (50 mM, pH 7.4) and adjusted to 10^7 spores per mL. 10 mL spore suspension was treated with 100 μg ethidium bromide (EtBr) for 20 mins following by 30 min-UV irradiation, which caused 90% killing of spores. Then the spores of the raw strain and the mutated spores were both spread onto the screening medium and incubated at 30°C for 3 days. *T. reesei* Rut C-30 spores were considered as the screening control.

Screening the Mutants with High Cellulase Production in Screening Medium

The hydrolysis halos of the mutants and the parent strain were observed. The mutants with larger size halos than *T. reesei* Rut C-30 in the screening medium were selected and inoculated into the fermentation medium. The crude enzyme of the original and mutant strains was both prepared.

Preparation of the Crude Enzyme

The spores from PDA were inoculated into PDB medium and incubated at 30°C and 120 rpm for 2 days. Then the 5% culture was inoculated to the fermentation medium and fermented for 6 days at 30°C and 120 rpm. The fermentation broth was centrifuged at 3000 g for 10 min. The supernatant was collected and filtered. The supernatant as crude enzyme was used for enzyme activity assay.

Determination of Enzyme Activity

The FPase, endoglucanase, xylanase and β- glycosidase activities were determined as reported (Gokhale, *et al*., 1988; Mehdi *et al*., 2010; Baljit *et al*., 2014). One unit (IU) of FPase and endoglucanase were defined as the amount of enzyme required to liberate 1 μmol of glucose, and one unit of xylanase and β-glycosidase enzyme activities were defined as the amount of enzyme required to liberate 1 μmol xylose or p-nitrophenol produced from the appropriate substrates/min of crude enzyme under the assay conditions (Gokhale, *et al*., 1988; Mehdi *et al*., 2010; Baljit *et al*., 2014).

Genetic Stability Evaluation

The genetic stability was determined by measuring the levels of enzyme production in a shake flask for successive generations. The desired mutant was maintained on PDA at 30°C. Then the mutants was inoculated to the PDB medium and cultured for 2 days. Then 5 mL culture was inoculated to the 100 mL fermentation medium and incubated for 6 days at 30°C and 120 rpm. The activities of crude enzyme were assayed. The genetic stability was determined for 10 generations.

The Time Course of Enzyme Production of *T. reesei* Rut C-30 and *T. reesei* EBUV-3

A comparison of cellulase production by *T. reesei* Rut C-30 and *T. reesei* EBUV-3 was developed. Briefly, the crude enzymes of both the strains were prepared and collected every 24 h during the fermentation progress and their enzyme activities including FPase, endoglucanase, xylanase and β-glycosidase were determined as described above. The fermentation lasted 9 days.

Kinetic Studies

The Michaelis-Menten kinetic parameters (K_m and V_{max}) were determined against *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and carboxymethyl (CM) cellulose as substrates, to detect catalytic efficiency of the raw and mutant strains, using Lineweaver-Burk plot (Baljit *et al*., 2014).

Table 1: Enzyme kinetics of β-glucosidase and CMCase produced by parent and mutant strains

Enzymes	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1} protein)
β-glucosidase (*T.reesei* Rut-30)	1.89±0.14	15.3±0.20
β-glucosidase (*T. reesei* VDE-3)	0.73±0.05	26.7±1.54
CMCase (*T. reesei* Rut-30)	2.28±0.20	22.8±1.01
CMCase (*T. reesei* VDE-3)	1.56±0.18	32.4±1.98

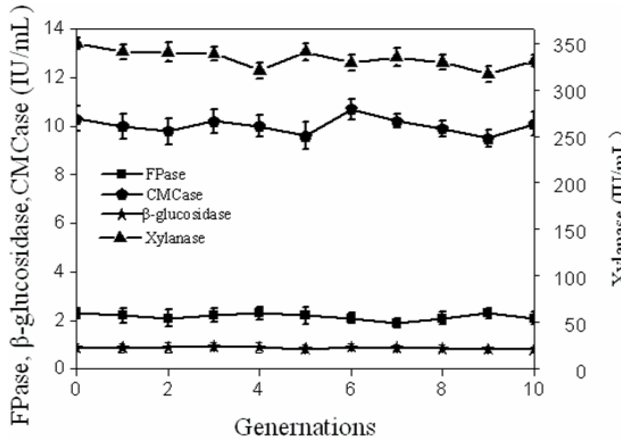

Fig. 1: Genetic stability analysis of cellulase activity for ten consecutive generations

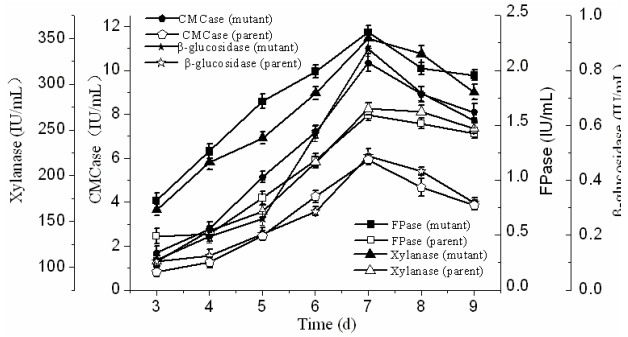

Fig. 2: The time course of the enzyme production of *T. reesei* Rut C-30 and *T. reesei* EBUV-3

Symbols: square, FPase; pentagon, CMCase; triangle, xylanase; star, β-glucosidase. Open, *T. reesei* Rut C-30; Solid, *T. reesei* EBUV-3

Saccharification Experiments

A typical hydrolysis mixture consisted of 1 g of steam-exploded corn stover, 20 mL of 50 mM sodium citrate buffer and the crude enzyme of *T. reesei* EBUV-3 or *T. reesei* Rut C-30. The mixtures were incubated in a rotary shaker at 120 rpm. To obtain the maximum reducing sugar, the saccharification conditions of *T. reesei* EBUV-3 were optimized including the reaction time, incubation temperature and the pH of reaction buffer. Then, the reaction production was heated to 100°C immediately to denature the enzymes, cooled and then centrifuged for 10 min at 8000 rpm. The supernatant was obtained for reducing sugar analysis, and the saccharification was calculated as follows:

Saccharification (%) = reducing sugars×0.9×100/ carbohydrates in substrate (Marimuthu *et al*., 2009).

Experimentation and Analysis

The values presented in graphs and tables are the means of three replications. Data were statistically analyzed by using analysis of variance (ANOVA) and expressed as mean. Significance was considered established at $P<0.05$.

Results

Mutation and Screening of Mutants for Cellulase

The spores of *T. reesei* Rut C-30 were applied to treatment with 10 μg/mL EtBr followed by UV irradiation, which caused 90% lethality. Firstly the selection was based on the diameter of hydrolysis halo surrounding the colony in the screening medium. Fifty mutants with larger size halos than the parent strain were picked up and further subjected to the next round screening. Briefly, the crude enzymes of the fifty mutants were generated and applied to assaying the ability of degrading the filter paper. Seven mutants showed approximately 1.1-1.7 folds increase in the activity of FPase. Among the seven mutants, *T. reesei* EBUV-3 showed the maximum FPase activity of 2.0 IU/mL, which was nearly 1.7-fold increase compared with the parent strain after 6-day fermentation.

Evaluation of Genetic Stability

T. reesei EBUV-3 was evaluated by measuring the enzyme activities for ten successive generations. It exhibited stable enzyme activities including FPase, CMCase, β-glucosidase and xylanase according to the result in Fig. 1.

Time Course of Cellulase Production of *T. reesei* Rut C-30 and *T. reesei* EBUV-3

T. reesei Rut C-30 and *T. reesei* EBUV-3 was separately fermented for 9 days and the fermentation broth was collected every 24 h. Then the FPase, endoglucanase, xylanase and β- glycosidase activities of the crude enzyme at intervals were measured. As shown in Fig. 2, both the strains released cellulases with 3 days of incubation. *T. reesei* Rut C-30 showed the maximum FPase (1.6 IU/mL), CMCase (5.9 IU/mL), β-glucosidase (0.5 IU/mL) and xylanase (273.3 IU/mL) activities on the seventh day of the fermentation. While the mutant *T. reesei* EBUV-3 reached the maximum enzyme activities of FPase (2.3 IU/mL), CMCase (10.3 IU/mL) and xylanase (349.9 IU/mL) on the seventh day. Interestingly, the maximum β-glucosidase activity was observed to be 1.0 IU/mL on the sixth day. Both the parent and mutant strains displayed similar fermentation tendency. On the 8th day the activities all began

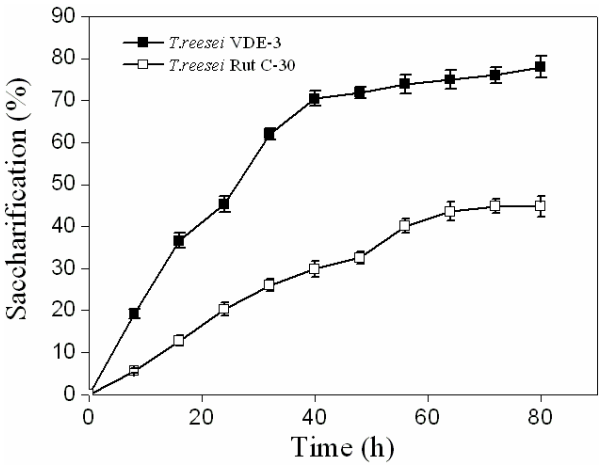

Fig. 3: Saccharification analysis of the fermentation liquor from *T. reesei* Rut C-30 and *T. reesei* EBUV-3

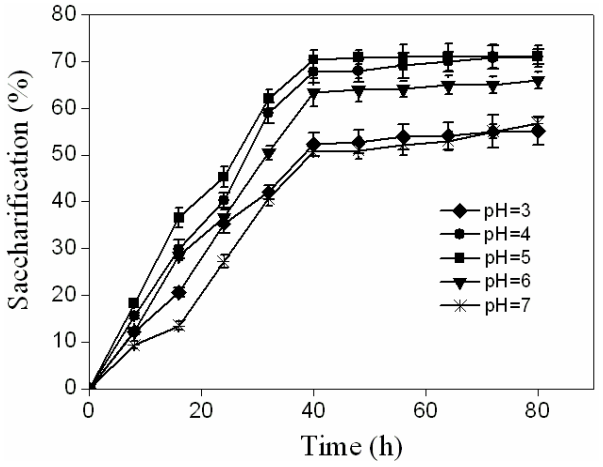

Fig. 4: The effect of pH on the saccharification of the fermentation liquor from *T. reesei* EBUV-3

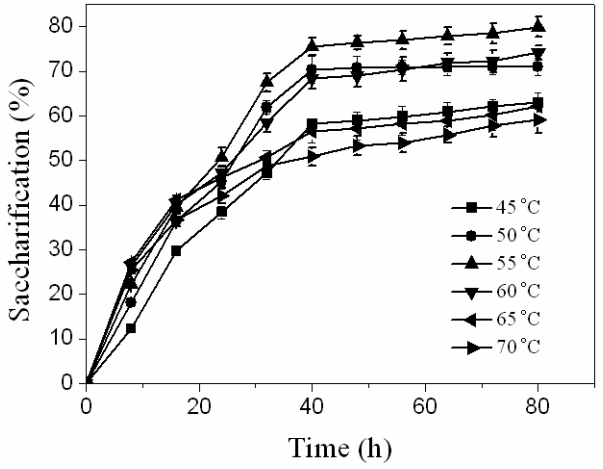

Fig. 5: The effect of temperature on the saccharification of the fermentation liquor from *T. reesei* EBUV-3

to decrease. From the results above, the maximum FPase, CMCase, β-glucosidase and xylanase of *T. reesei* EBUV-3 have increased 1.4, 1.7, 2.0 and 1.3 folds, respectively.

Kinetic Studies

We analyzed kinetics of β-glucosidase and CMCase produced by *T. reesei* Rut C-30 and *T. reesei* EBUV-3. The results in Table 1 clearly suggested higher rate of catalysis of β-glucosidase and CMCase in *T. reesei* EBUV-3 thus exhibiting significantly lower K_m and higher V_{max} values.

Saccharification Analysis of *T. reesei* Rut C-30 and *T. reesei* EBUV-3

The corn stover has been recognized to be an ideal inducer to produce sugars. We tried to produce sugars from steam-exploded corn stover in a small volume reaction including 1 g of steam-exploded corn stover, 20 mL of 50 mM sodium acetate buffer (pH 5.0) and the crude enzyme of *T. reesei* EBUV-3 or *T. reesei* Rut C-30 at 50°C. Yield of reducing sugars was determined by DNS method. The saccharification level of *T. reesei* EBUV-3 was significantly higher than *T. reesei* Rut C-30 (Fig. 3). After nearly 48 h the reducing sugars produced by *T. reesei* EBUV-3 reached stable level, it showed 64.7% saccharification; meanwhile, *T. reesei* Rut C-30 only displayed 46.1% saccharification after 56 h (Fig. 3).

The Effect of pH on the Saccharification

To determine the pH profile of the cellulase produced by *T. reesei* EBUV-3, the saccharification activities at various pH values from 3.0 to 7.0 were examined at 50°C. The maximum saccharification activity was determined at an optimal pH of 5.0 (Fig. 4).

The Effect of Temperature on the Saccharification

The effect of temperature on the saccharification was determined ranging from 45°C to 70°C at pH 5.0. *T. reesei* EBUV-3 showed the maximum saccharification with an optimal temperature of 55°C (Fig. 5). 55°C might be a suitable temperature for the cellulase system including endoglucanase, cellobiohydrolase and β-glucosidase to saccharify the steam-exploded corn stover. According to the results of the effect of pH and temperature on the saccharification of *T. reesei* EBUV-3, *T. reesei* EBUV-3 displayed nearly 70% saccharification on the optimal conditions after 48 h.

Discussion

Lignocellulosic biomass, such as corn stover, is served as a widely abundant and renewable feedstock for the production of sugar. The economic cellulosic sugar production is related several key technological issues, such as efficient pretreatment, efficient utilization of feedstock and

hydrolysis efficiency. Yu have reported the enzymatic saccharification of corn stover was enhanced with sequential fenton pretreatment (He *et al*., 2015). Liu improved the saccharification of corn stover with exploded corn stover (Liu and Chen, 2016). However, such methods will increase the cost of the saccharification process. Use of mutagensis-selection of microorganisms has been found effective in increasing enzyme production (Sanjeev *et al*., 2014). Our results showed that only seven of the fifty mutants exhibited higher FPase activity than the parent strain. Mutant selection could not only depend on the hydrolysis halo selection. Larger hydrolysis halos displayed might cause by the larger colonies. FPase activity measurement with the crude enzyme obtained from almost equivalent biomass of the mutants might be more reliable. Hence, multiple-selection was a necessary and reliable method as described by other reports (Sanjeev *et al*., 2014; He *et al*., 2009).

The enzyme production of *T. reesei* EBUV-3 could be further optimized by the optimization of fermentation medium and culture conditions as other reports (Namita *et al*., 2012; Ketna *et al*., 2013; Sanjeev *et al*., 2014). The maximum FPase, CMCase, β-glucosidase and xylanase of *T. reesei* EBUV-3 have increased 1.4, 1.7, 2.0 and 1.3 folds, respectively. The data suggested that *T. reesei* EBUV-3 was potential to be an efficient cellulase producer in the industrial application. The characterization of the cellulase from *T. reesei* EBUV-3 biased acid condition. Xu have also reported similar findings (Chen *et al*., 2013; Baljit *et al*., 2014; Sasikumar *et al*., 2014; Xu *et al.*, 2014).

Conclusion

In conclusion, *T. reesei* EBUV-3, the UV and EtBr mutant from the raw *T. reesei* Rut C-30, was identified to be a higher cellulase producer. This mutant showed nearly 1.4, 1.7, 2.0 and 1.3 folds enhancement of the activities of FPase, CMCase, β-glucosidase and xylanase compared with *T. reesei* Rut C-30. Particularly, the β-glucosidase and CMCase from *T. reesei* EBUV-3 exhibited higher substrate affinity and reaction rate. Moreover, the saccharification of steam-exploded corn stover after 40 h by *T. reesei* EBUV-3 increased 24%. Thus, *T. reesei* EBUV-3 could be a potential candidate of the production of cellulase for the sacchrification of cellulosic biomass.

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