Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production

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Abstract The strain of *Trichoderma reesei* Rut C-30 was subjected to mutation after treatment with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NG) for 6 h followed by UV irradiation for 15 min. Successive mutants showed enhanced cellulase production, clear hydrolysis zone and rapid growth on Avicel-containing plate. Particularly, the mutant NU-6 showed approximately two-fold increases in activity of both FPA and CMCase in shake flask culture when grown on basal medium containing peptone (1%) and wheat bran (1%). The enzyme production was further optimized using eight different media. When a mixture of lactose and yeast cream was used as cellulase inducer, the mutant NU-6 yielded the highest enzyme and cell production with a FPase activity of 6.2 U ml⁻¹, a CMCase activity of 54.2 U ml⁻¹, a β -glucosidase activity of 0.39 U ml⁻¹, and a fungal biomass of 12.6 mg ml⁻¹. It deserved noting that the mutant NU-6 also secreted large amounts of xylanases (291.3 U ml⁻¹). These results suggested that NU-6 should be an attractive producer for both cellulose and xylanase production.

Keywords Cellulase · *Trichoderma reesei* · NTG and ultraviolet mutation · Fermentation

Introduction

Cellulose is the most abundant renewable carbon resource on earth. It is synthesized mainly by plants and together with hemicelluloses, lignin and pectin constitutes most of the plant cell wall material. Even though a large amount of cellulose is formed annually, its amount on earth does not accumulate due to fungi and bacteria efficiently degrading these materials to provide themselves with carbon and energy source for growth and recycling carbon back into the ecosystem. The efficient degradation of cellulose is a complex process involving the synergistic action of a number of cellulolytic enzymes [1]. In recent years, the cellulolytic enzymes has become an industrially important enzyme that is used in various applications, such as in the textile industry [2, 3], the food and feed industry [4] and the pulp and paper industry [5]. In addition, the growing concerns about the shortage of fossil fuels and air pollution caused by incomplete combustion of fossil fuel have also led to an increasing focus on production of bioethanol from lignocellulosis and especially the possibility to use cellulases to perform enzymatic hydrolysis of the lignocellulosic materials [6, 7]. In order to make those large-scale applications economically feasible, the cost of the cellulolytic enzymes needs to be reduced [8, 9].

The fungus *Trichoderma reesei* has been shown to secrete large amounts of efficient cellulolytic enzymes [10], which render it an attractive strain for industrial production. The extracellular cellulolytic system of *T. reesei* is composed of 60–80% cellobiohydrolases or exoglucanases (EC3.2.1.74), 20–36% of endoglucanases (EC3.2.1.4) and 1% of β -glucosidases (EC3.2.1.21), all of which act synergistically in the conversion of cellulose into glucose [7, 11].

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The strain of Rut C-30 has been subjected to mutagenesis and genetic modification to increase the enzyme production [12–14]. However, despite the efforts of those laboratories, no commercially efficient enzyme complex has been produced. In addition, most of the *T. reesei* cellulase system is deficient in cellobiase, resulting in the accumulation of the disaccharide cellobiose, which causes repression and end product inhibition for enzyme biosynthesis [7]. Thus, the conversion of waste cellulose to glucose is still not commercially feasible. To resolve these problems, many researches have been involved in isolating new microorganisms to produce cellulolytic enzymes with higher specific activity and greater efficiency [15]. Other approaches such as genetic modification and heterologous expression are also to be utilized [16].

The aim of the present study was to improve the cellulase production of *T. reesei* Rut C-30 through a successive mutagenesis step. The optimization of the enzyme production in shake flasks has also been described.

Materials and methods

Microorganisms and media

The strain of *T. reesei* Rut C-30 was obtained from the Center of National Collection for Industrial Microorganism (CICC), Beijing, China. The parent and mutant strains were maintained on potato dextrose agar (PDA) and sub-cultured once in every three months. The PDA (per liter) consisted of extract from 300 g of potatoes, 20 g of glucose, and 20 g of agar. Basal medium (BM) (per liter) consisted of 1 g of KH_2PO_4 , 5 g of MgSO_4 , 0.1 g of CaCl_2 , 0.1 g of NaCl , 0.05 g of FeSO_4 , 0.02 g of MnSO_4 , and 0.02 g of ZnSO_4 . The initial pH of BM was adjusted to 5.5 using 1 N NaOH. The BM supplemented with different carbon (1%) and nitrogen (1%) sources were used as the fermentation medium. The selection medium (per liter) consisted of 5 g of Triton X100, 20 g of agar and 5 g of phosphoric acid-swollen cellulose. For long-term storage, all stock cultures were maintained at -80°C in 20% glycerol.

Preparation of mutants

T. reesei Rut C-30 (parent strain) was grown on PDA for 14 days at 28°C . Conidia generated on slant are transferred into 10 ml BM with 0.1% Tween-80 and cell count was adjusted to 10^7 spores ml^{-1} . NG (20 mg) was added to 10 ml suspension. This suspension was kept at room temperature for 6 h followed by UV irradiation for 15 min (15 cm apart from lamp), which caused 99% killing of spores. Then the treated suspension (100 μl) was spread on screening plates

and incubated at 28°C for 7 days. The mutants were primarily selected on the basis of clearance zones appeared on the plate. To confirm the primary selection, all promising mutants (approximately 10^7 spores) were transferred into 100 ml shake flasks containing 30 ml of fermentation medium (BM supplemented with peptone (1%) and wheat bran (1%), respectively). The flasks were incubated at 28°C with shaking at 200 rpm. After 72 h fermentation, the samples were removed and the supernatant was analyzed for filter paper cellulase, endoglucanase, β -glucosidase and xylanase activities

Optimization of enzyme production in shake flasks

To optimize the enzyme production for the most desired mutants, the experiments were carried out in 500 ml Erhlenmeyer flask with 100 ml of fermentation medium containing different carbon and nitrogen sources (initial culture pH 5.0). The flasks were inoculated with spores (approximately 10^7) from 10-days-old culture grown on PDA plates and incubated at 28°C with shaking at 200 rpm. The samples were removed at various time intervals and centrifuged at 3,000g for 10 min. The supernatant was analyzed for extracellular enzyme activities and soluble protein. To elucidate the influences of initial pH on enzyme production, the fermentation was performed at optimal temperature using the BM (supplemented with 1% yeast cream and 1% lactose, respectively) with a pH range of 3.0 to 8.0.

Determination of enzyme activities

The culture supernatants (used as the enzyme sources) were separated from the cellular mass by centrifugation for 5 min at $4,000\times g$. Filter paper cellulase (FPA), endoglucanase, β -glucosidase and xylanase activities were determined as reported earlier [17, 18]. FPase was assayed by incubating 0.1 ml suitable diluted enzyme (10-fold) with 50 mg of filter paper (Whatman no.1) in 1.9 ml citrate buffer. The reaction mixture was incubated at 50°C for 60 min. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars in 1 min at 50°C . Endoglucanase (CMCase, Endo-1,4- β -D-glucanase; EC3.2.1.4) activity was assayed in the total reaction mixture of 1 ml containing 0.5 ml of diluted enzyme and 0.5 ml of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5). This reaction mixture was incubated at 50°C for 30 min. The β -glucosidase activity was determined by incubating 0.1 ml diluted enzyme with 0.5 ml acetate buffer (50 mM, pH 5.0) containing 2.5 mg cellobiose. After incubation at 50°C for 10 min, the glucose released was measured by the glucose oxidase peroxidase method (Sigma). The xylanase activity assay was based on the method described by Bailey et al.

[19], with 1% oat spelt xylan (Sigma) as the substrate. The amount of released sugar was determined by the dinitrosalicylic acid method described by Miller et al. [20].

Determination of biomass

The biomass was measured as myceliar protein. Cultures were grown in 250 ml conical flasks containing 50 ml of fermentation medium (BM with 1% yeast cream and 1% lactose). At the desired time intervals, the content of a flask was centrifuged at 5,000 g for 10 min. The pellet was washed, resuspended in cold saline and homogenized by ultrasonic treatment. An aliquot of the homogenized suspension was extracted with one volume of 1N NaOH at 50°C for 45 min and centrifuged. The supernatant was precipitated with 20% TCA, and the protein content was determined by the Bradford assay [21], using bovine serum albumin as a standard. The dry fungal mass was determined after drying the cell pellet at 40°C until constant weight [22].

Hydrolysis experiments

The enzyme preparations for hydrolysis of Avicel and wheat bran were derived from fermentation cultures in shake flask. The hydrolysis of the substrates was carried out in 100 ml conical flask containing 50 ml citrate buffer (pH 5.0, 50 mM), 2.5 g Avicel or wheat bran, 5 mg sodium azide and crude enzyme preparation. The hydrolysis was performed for 6 days at 40°C, with a stirring rate of 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals [8].

Characterization of the enzyme

For enzyme characterization, the culture supernatant was filtered and concentrated (about 15-fold) in a Diaflo Ultrafilter PM10 concentrator (Amicon). The temperature optimum of the enzyme was measured by performing the enzyme activity assay at temperatures ranging from 20°C to 90°C. Thermostability was tested by heating enzyme samples for different times at various temperatures, and the activity was assayed at 50°C for 10 min. Assays at different pH values were performed at the optimal temperature over a pH range of 3.0 to 9.0. The buffer used were 50 mM citrate (pH 3.0), 50 mM citrate phosphate (pH 4.0 to 7.0) and 50 mM phosphate (pH 8.0), respectively.

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

plates at 28°C, and the intervals for each generation were 14 days.

Experimentation and analysis

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

Results

Mutation of *T. reesei* Rut C-30

The strain of *Trichoderma reesei* Rut C-30 was subjected to mutation after treatment of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by UV irradiation. The primary selection of hyperproducing mutants was based on the diameter of clearing zone surrounding the colony on the screening media. The screening media gave fairly reliable indication of increased cellulolytic activities. After mutagenic treatment, enzyme production by the selected mutants was determined in shake flask cultures, and the most promising strain was used for further study. In the primary selection, 47 colonies were selected on screening plates. These mutants were further assessed for both cellulases and xylanases production in shake flasks containing BM with 1% peptone and 1% wheat bran. Among them, seven mutants showed enhanced both FPA and CMCase activities (Table 1). The mutant NU-6 showed approximately 1.8 fold increases in activity of FPase (4.7 U ml⁻¹) and 2.4 fold increases of CMCase (44.2 U ml⁻¹) in shake flask culture when grown on basal medium containing 1% peptone and 1% wheat bran (Table 1). It also showed a high level of xylanases production (268.1 U ml⁻¹). But the mutant secretes less β -glucosidase (0.32 U ml⁻¹) than the parent strain.

Optimization of enzyme production by different media

The mutant NU-6 was further investigated for both cellulase and xylanase productions in shake flasks containing optimized basal medium with different carbon and nitrogen sources (Table 2). In this study, the mutant grew very well in BM supplemented with wheat bran (1%) and peptone (1%) as the carbon and nitrogen sources, respectively. The highest mycelium biomass (13.2 mg ml⁻¹) was obtained from this medium. When lactose was used as the inducer, the mutant NU-6 produced a maximal FPase activity of 6.2 U ml⁻¹, and a CMCase activity of 54.2 U ml⁻¹. However,

the use of inorganic nitrogen sources such as $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl and urea led to a very poor growth rate, which subsequently resulted in a reduced enzyme production.

The profiles of enzyme production

The time course of enzyme production by mutant NU-6 in shake flask was shown in Fig. 1. The mutant secreted maximum FPase, β -glucosidase and xylanase activities after 72 h of fermentation. However, the maximum CMCase activity was found after 96 h. The maximal cell dry mass (12.4 mg ml^{-1}) was obtained after 96 h (Fig. 2). The specific activities for all enzymes decreased as the maximum activity was achieved. Because the presence of insoluble substrates (such as cellulose) may complicate the determining of cell weight, the soluble inducer (lactose) was used in this study.

Influences of temperature and pH on enzyme production

To evaluate the influences of temperature and pH on enzyme production, the mutant was cultured in BM with lactose (1%) and yeast cream (1%) as the carbon and nitrogen sources, respectively. The optimal temperature for enzyme production was about 30°C . Both xylanase and cellulase activities significantly decreased when the fermentation was performed over 40°C or below 20°C (Fig. 1C). The highest FPase and CMCase activities were observed at pH 5.0, however, the highest xylanase activity was observed at pH 5.5 (Fig. 1D).

Enzymatic hydrolysis

The enzyme preparations produced by mutant NU-6 has been used to hydrolyze cellulosic substrates at 5% concen-

Table 1 Comparison of enzyme production of *T. reesei* Rut C-30 and its mutants

Strain	Enzyme activities (U ml^{-1}) ^a			
	FPase	CMCase	β -glucosidase	Xylanase
Parent	2.6 ± 0.2^a	18.2 ± 1.7^a	0.35 ± 0.06^a	212.4 ± 11.3^a
NU-1	4.1 ± 0.3^b	35.3 ± 2.8^b	0.36 ± 0.04^a	135.8 ± 9.5^b
NU-2	2.9 ± 0.2^{ac}	22.1 ± 3.2^c	0.28 ± 0.03^b	168.9 ± 6.8^b
NU-3	3.2 ± 0.3^c	27.5 ± 3.6^d	0.47 ± 0.03^{ac}	245 ± 14.7^{ac}
NU-4	3.9 ± 0.3^b	36.4 ± 2.5^b	0.32 ± 0.06^a	85.2 ± 6.2^d
NU-5	3.4 ± 0.2^c	28.6 ± 1.9^d	0.23 ± 0.02^b	59.3 ± 5.1^e
NU-6	4.7 ± 0.5^d	44.2 ± 2.2^e	0.32 ± 0.04^{ac}	268.1 ± 10.2^c
NU-7	3.1 ± 0.3^c	24.3 ± 2.7^c	0.36 ± 0.03^a	234.7 ± 13.6^a

^a The cultures were grown in BM containing peptone (1%) and wheat bran (1%), respectively. The values of enzyme activities given in the table are the average of three independent experiments.

^b The data were compared by ANOVA test and expressed as mean $\pm$ SD.

^c Means in a line followed by different letters are significant at $p < 0.05$.

Table 2 Comparison of enzyme production of mutant NU-6 cultivated in different fermentation media

Medium	Enzyme activities (U ml^{-1})				
	FPase	CMCase	β -glucosidase	Xylanase	Biomass
BM+YC (1%) + wheat bran (1%)	3.9 ± 0.3^a	38.9 ± 4.5^a	0.27 ± 0.03^a	280.6 ± 14.9^a	10.3 ± 1.1^a
BM+YC (1%) + lactose (1%)	6.2 ± 0.5^b	54.2 ± 3.8^b	0.39 ± 0.03^b	291.3 ± 12.8^a	12.6 ± 0.8^b
BM+YC (1%) + CMC (1%)	4.6 ± 0.4^c	46.7 ± 2.9^c	0.40 ± 0.02^b	25.3 ± 2.1^b	12.3 ± 2.1^b
BM+YC (1%) + xylan (1%)	3.6 ± 0.3^a	41.2 ± 3.4^c	0.35 ± 0.03^c	324.5 ± 16.2^c	9.5 ± 0.6^a
BM+WB (1%) + peptone (1%)	4.8 ± 0.5^c	45.1 ± 3.2^c	0.42 ± 0.03^b	276.2 ± 13.2^a	13.2 ± 1.2^b
BM+WB (1%) + urea (1%)	ND	ND	ND	ND	1.4 ± 0.1^c
BM+WB (1%) + $(\text{NH}_4)_2\text{SO}_4$ (1%)	1.7 ± 0.2^d	12.1 ± 1.7^d	0.11 ± 0.02^d	27.2 ± 3.2^b	7.2 ± 0.4^d
BM+WB (1%) + NH_4Cl (1%)	1.4 ± 0.1^d	13.8 ± 2.1^d	0.13 ± 0.02^d	69.8 ± 5.7^d	8.3 ± 0.7^d

^a The values of enzyme activities given in the table are the average of three independent experiments.

^b The data were compared by ANOVA test and expressed as mean $\pm$ SD.

^c Means in a line followed by different letters are significant at $p < 0.05$.

^d Biomass was expressed by the unit of mg ml^{-1} .

tration. Yield of enzymatic hydrolysis was highest when Avicel was used as the substrate (Fig. 2B). The wheat bran hydrolysis was lower than Avicel, but both of them linearly increased with the increasing of reaction time.

Characterization of the enzyme

Enzymatic assays at different temperatures revealed that both FPA and CMCase have an optimal activity at 50°C (Fig. 3A). Concerning the effect of the pH, FPA and

CMCase showed an optimal activity at pH 5.0 and 6.0, respectively. When the pH was below 3.0 or above 9.0, only 20%–30% of the maximum activity was reached. Both FPA and CMCase were stable at 50°C, and the total activity retained more than 80% after 30 min incubating at this temperature (Fig. 3C, D).

Evaluation of genetic stability

The genetic stability of NU-6 was evaluated by measuring the enzyme activity for seven successive generations. As

Fig. 1 Extracellular cellulolytic enzyme [Filter paper activity (□), β-glucosidase (□), CMCase (▲) and Xylanase (●)] production of NU-6 grown in BM supplemented with yeast cream (1%) and lactose (1%). (A) Influence of incubation time on enzyme production (U mL⁻¹); (B) Influence of incubation time on specific enzyme production (U mg⁻¹ cell); (C) Influence of temperature on enzyme production; (D) Influence of initial pH on enzyme production. For clarity purposes, standard deviations have not been added for this figure

Fig. 2 Profile of biomass accumulation and hydrolysis of cellulolytic substrates. (A) Biomass accumulation (mg ml⁻¹) [Dry biomass (□); Soluble proteins (□)]. (B) The hydrolysis of Avicel (□) and wheat bran (□) using enzyme preparations of NU-6.

Fig. 3 Characterization of the recombinant Xyn2. (A) Effect of temperature on the activity of FPA (▲) and CMCase (○); (B) Effect of pH on the activity of FPA (▲) and CMCase (○); (C) The temperature stability was determined after preincubating the enzyme in the absence of the substrate for 30 min at 40, 50, and 60°C. (D) The thermostability was determined by preincubating the enzyme at optimal temperatures in the absence of substrate for 5, 10, 15, 20, 25, and 30 min before measuring its activity. The activity prior to the preincubations at different temperatures was taken as 100%.

shown in Fig. 4, the mutant NU-6 is quite stable for both cellulases and xylanase production.

Discussion

In recent years, enzymatic degradation of cellulosic materials by fungal enzyme systems has been suggested as a feasible alternative to produce fermentable sugars and fuel ethanol from lignocellulosics [23, 24]. Thus, cellulases produced by fungi, especially by *Trichoderma reesei* and *Trichoderma viride* have been most extensively studied. The *T. reesei* is a common soil fungus of the *Deuteromycete* family which has been widely used for the commercial cellulases and xylanases production. In order to reduce the price, people have developed many approaches to increase enzyme yields, such as genetic modification, heterologous expression and fermentation optimizing [12, 13, 25]. However, the strain improvement by mutation is still a traditional and efficient method to enhance the enzyme production [8, 26]. In this study, we adopted the mutation steps involving treatment of *T. reesei* spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 6 h followed UV-irradiation for 15 min, where we observed 99% lethality. We successfully isolated one mutant, NU-6, which showed a larger zone of cellulose

hydrolysis. Enzyme assay verified that the mutant showed approximately 1.8-fold increases in activity of FPase and 2.4 fold increases of CMCase in shake flask culture. The parent strain Rut C-30 is a mutant isolated in mutant selection programs at Rutgers University, Rutgers, NY. [27], which started with *T. reesei* QM6a (Fig. 5). As a result, the strain Rut C-30 produces approximately four times more cellulase than the original parent (QM6a). However, both the original parent and mutants (NU-6, Rut C-30) produces few β -glucosidase.

Previous study revealed that the cellulase is an inducible enzyme and several carbon sources have been found to efficiently promote enzyme production [28–30]. Cellulose itself has been recognized as one of the best inducers. Other ideal inducers include saphorose and lactose [31, 32]. In this study, we optimized the enzyme production using basal medium supplemented with different substrates (carbon and nitrogen sources). The highest activities of FPase (6.2 U ml⁻¹) and CMCase (54.2 U ml⁻¹) were obtained when lactose (1%) and yeast cream (1%) was supplemented into the basal medium. The cheap raw material such as wheat bran has been also reported to be an ideal inducer [8]. The mutant NU-6 grew very well and produce large amounts of enzymes in basal medium supplemented with wheat bran and organic nitrogen sources (such as peptone). However, the combina-

Fig. 4 Genetic stability analysis for seven consecutive generations [Filter paper activity (□), β-glucosidase (□), CMCase (▲) and Xylanase (●)]; The mutant NU-6 was fermented in BM supplemented with yeast cream (1%) and lactose (1%).

Fig. 5 Lineage of *T. reesei* Rutgers and VTT-D series mutants derived from the wild type *T. reesei* QM 6α. Mutagenic agents: NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; γ, gamma irradiation; dES, diethyl sulfate

tion of wheat bran and inorganic nitrogen sources such as urea, $(\text{NH}_4)_2\text{SO}_4$ and NH_4Cl acutely decreased the enzyme production (Table 2). These results are in good agreement with previous findings that *T. reesei* grows rapidly in nitrogen-rich sources such as peptone, but more slowly is ammonium salts [1, 33].

It is evident that the highest cellulase activity was obtained after 72 h fermentation (Fig. 1A). Both cellulases and xylanase activity significantly decreased after 96 h. This may be attribute to the substrate consumption. Another important reason is the catabolite repression caused by cellobiose. Both the mutant and parent strain produce few β-glucosidase, therefore cellobioses accumulate and repress enzyme biosynthesis [34].

Earlier reports revealed that the fermentation temperature and pH are two important factors affecting the biosynthesis of enzymes. The biosynthesis of cellulases by parent strain Rut C-30 was favored by a lower pH (4.0), whereas the production of xylanases was favored by a higher pH (7.0) [35]. In this study, the highest cellulases and xylanase activities were observed at pH 5.0 and 5.5, respectively. Although the temperature optimum for cell propagation was about 25°C, the temperature optimum for enzyme biosynthesis was found to be 30°C. An elevated temperature resulted in a reduced enzyme production. Similar results were obtained from other mutants such as *Trichoderma harzianum* E58 [36], *Trichoderma reesei* Rut C-30 [37] and *Trichoderma aureoviride* 7-121 [7]. In addition to measuring the enzyme activities, it was also very important to determine the hydrolytic capacity of the produced enzymes. More than 60% of hydrolysis was obtained when Avicel (5%) was used as the substrate, whereas less than 40% hydrolysis was obtained from wheat bran. The decreased hydrolysis could be due to the fact that wheat bran was much more difficult to dissolve. In this study, the produced enzyme was fully characterized. As indicated in Fig. 3, both FPA and CMCase have a moderate thermostability and inactivated rapidly above 60°C. However, they retained at least 80% of its total activity after 30 mins incubating at 50°C. The enzyme was active over a broad range of pH 3.5–8.0 (retained more than 60% activity). These attributes should make the enzyme a suitable candidate for various applications, such as bleaching application in the pulp and paper industry.

The evaluation of genetic stability showed that the mutant NU-6 is quite stable for both cellulases and xylanase production (Fig. 4). Coupled with its enhanced enzyme yields, we are convinced that this mutant should be an attractive strain for industrial enzyme production. Because the mutant NU-6 still secretes few β-glucosidase, the isolating of a new wild-type strain with much better original β-glucosidase yields might be one alternative way for a new mutation program. Furthermore, the mutant NU-6 detailed in this study is also available for further mutation programs.

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