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Efficient Isolation and Characterization of a Cellulase Hyperproducing Mutant Strain of *Trichoderma reesei*[S]

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A cellulase hyperproducing mutant strain, JNDY-13, was obtained using the ARTP mutation system and with *Trichoderma reesei* RUT-C30 as the parent strain. Whole-genome sequencing of JNDY-13 confirmed that 105 of the 653 SNPs were point mutations, 336 mutations were deletions and 165 were insertions. Moreover, 99 mutations were insertions and duplications. Among all the mutations, the one that occurred in the galactokinase gene might be related to the production of cellulases in *T. reesei* JNDY-13. Moreover, the up-regulation of cellulase and hemicellulase genes in JNDY-13 might contribute to higher cellulases production. Under optimal conditions, the highest cellulase activity by batch fermentation reached 4.35 U/ml, and the highest activity of fed-batch fermentation achieved was 5.40 U/ml.

Keywords: Cellulase, *Trichoderma reesei*, ARTP-LiCl, NGS, galactokinase

Introduction

Cellulose and hemicellulose polysaccharides are β-(1,4)-linked glucose polymers with diverse compositions. They make up 60–80% of the plant cell wall, and they are derived from photosynthesis and the absorption of carbon dioxide by plants [1]. Therefore, the conversion of cellulosic and hemicellulosic polymers into bioethanol or platform chemicals is considered important for the attempt to reduce carbon dioxide emissions and alleviate the overdependence on fossil fuels [2]. Cellulases produced by certain filamentous fungi, such as *Trichoderma reesei*, are considered the most optimal enzyme compound to thoroughly decompose cellulose into glucose. Glucose, in turn, can be utilized to synthesize other valuable chemicals and energy. Cellulases have extensive applications in animal feed, textiles, grain alcohol fermentation, pulp and paper processing, starch processing, and the pharmaceutical,

malting, and food industries [3–5].

Sixteen hemicellulolytic and ten cellulolytic enzyme-encoding genes were identified in *T. reesei* through a genome-wide analysis [6]. *T. reesei* Rut-C30 is the most extensively studied strain [7, 8]. It evolved from the wild-type strain *T. reesei* QM6a through three rounds of mutagenesis (N-nitroguanidine mutagenesis and ultraviolet (UV) light mutagenesis), which was followed by a screening for the release from carbon catabolite repression (CCR) and high production of cellulases [9–11]. Furthermore, genetic and phenotypic changes in *T. reesei* Rut-C30 have been extensively studied [8, 12]. Consequently, many details about the catalytic function and molecular structure of these cellulases and hemicellulases have already been illuminated, and several aspects of the regulatory mechanism of their secretion have also been elucidated.

The ARTP (atmospheric and room temperature plasmas) mutation system is a novel mutagenesis technology, and it

has been widely applied in mutation breeding of microorganisms (*e.g.*, fungi, actinomycetes, and bacteria). This enhances the production of industrial products as a consequence of its superior characteristics, which include operational safety, lower costs, and low plasma temperature [13, 14]. The plasma can be easily generated, and it can destroy a DNA strand instantaneously, both at room temperature and atmospheric pressure. As a result, various kinds of breakages of oligonucleotides and plasmid DNA are generated with different plasma dosage [15–19].

In this study, ARTP was employed to gain a mutant *T. reesei* strain with high cellulase activity. The mutant strain was analyzed by next-generation sequencing, and transcription levels of key genes closely related to cellulases and hemicellulases were characterized by Reverse Transcription-quantitative PCR. Finally, the fermentation medium and process were optimized to achieve high cellulase activity.

Materials and Methods

Strain and Culture Conditions

T. reesei RUT-C30 was used as the parental strain, and it was grown in 250 ml shake flask at 30°C on a rotary shaker (200 rpm). The seed medium was prepared with 20 g/l lactose [20]. Composition of the fermentation medium was as follows (1 L): 10 g lactose, 10 g avicel, 12 g corn steep liquor, 1.5 g $(NH_4)_2SO_4$, 1 g $MgSO_4$, 1 g $CaCl_2$, 2 ml Tween 80, and 1 ml Mandels solution [21]. The inoculum was prepared by transferring *T. reesei* RUT-C30 spores into a 500 ml shake flask containing 300 ml of seed medium. The seeds were grown in a shake flask at 30°C at 200 rpm for 3 days, and then transferred to a bioreactor (5-L bioreactor, Bailun Shanghai). The fermentations were carried out at 30°C with aeration of 1 vvm and a stirring speed of 400 rpm. The pH was maintained at 5.0 by supplementing 2 M NaOH or 2 M H_2SO_4. When performing fed-batch fermentation, the concentrated lactose (125 g/l) and $(NH_4)_2SO_4$ (27.5 g/l) were fed into the bioreactor during the fermentation. The biosamples were taken regularly and treated through a suction filter. The filtrate was collected for subsequent analysis.

Screening of Hyperproducing Mutants by ARTP

The ARTP mutation system was manufactured by Tsinghua University [19]. The working radio frequency (RF) power input was 100 W, and the gas flow volume was 10.0 SLM (standard liter per minute). Ten μl of fresh spore (spore concentration: 10^{7-8}/ml) was dispersed onto a sterilized plate (12 mm in diameter), and then the plate was put into a vessel with a fixed treatment distance of 2.0 mm. The treatment time varied from 40 to 260 sec. To resuspend the spores, the plate was immediately transferred to sterile saline and shaken for 30 sec after mutation treatment. With appropriate dilution, 200 μl of spore suspension was well-distributed onto selective agar plates that contained 10 g/l carboxymethyl cellulose sodium (CMC), 3 g/l lactose, and 1 g/l lithium chloride. Afterwards, the plates were cultured in the mold incubator for 4 days at 30°C. Then, the plates were dyed with Congo red for 30 min and washed with sterile NaCl solution. Colonies that were surrounded with large halos on the plates were chosen as the original strains for the next mutagenesis cycle [22]. The H/C number was defined as the diameter of the halo produced by hydrolysis divided by the diameter of the colony. Since the wild type H/C varies from 1.02 to 1.26, an H/C > 1.3 indicates that the mutagenesis was positive and an H/C <1 indicates that the mutagenesis was negative.

Measurement of Cellulose Content, Fungal Biomass, and Lactose Consumption

To determine the cellulose content of the cultures, 10 ml of sample was centrifuged (3,000 g, 20 min). Then, the pellets were resuspended in 3 ml of acetic acid-nitric acid solution (made of 15 ml of nitric acid with 150 ml of 80% acetic acid). Next, they were incubated in a water bath for 30 min before being cooled down and centrifuged (3,000 g, 20 min). Afterwards, the pellets were washed with an equal amount of distilled water. The remaining solid was primarily cellulose, and it was dried to constant weight at 40°C under reduced pressure [23]. The measured value of the residual cellulose content was an average of three replicates. The fungal biomass concentration was measured by filtration [24]. The biomass was washed with an appropriate amount of water and dried on filter paper at 105°C to a constant weight. The dry biomass weight was calculated as the difference between the total dry weight (containing cellulose and mycelium) and the residual cellulose weight.

The residual lactose was measured by HPLC (higher performance liquid chromatography) (Bio-Rad HPX-87P ion exclusion column) [25]. Samples used for analysis were stored at −20°C before analysis.

Cellulase and Galactokinase Assays

Filter Paper Unit (FPU) was chosen to measure the cellulase activity, according to the IUPAC protocol [26]. For galactokinase activity analysis, an HPLC-based method was applied to measure the concentration of galactose-1-phosphate in 1 ml of the reaction solution, which contained 10 mM ATP, 20 mM D-galactose, 0.7 ml crude extract, and 10 mM $MgSO_4$ (in 0.1 M phosphate buffer, pH 7.6). Specifically, 0.2 g of fresh wet mycelia was added into 2 ml of 0.1 M phosphate buffer, and the crude extract was obtained after ultrasonic homogenization in an ice bath. The enzymatic reaction was initiated by the addition of D-galactose, and it was incubated at 37°C for 30 min. Then, the mixture was put on ice to terminate the reaction. To remove the SO_4^{2-}, it was precipitated by adding an equal molar amount of $Ba(OH)_2$. Then, the mixture was centrifuged at 8,500 g for 5 min. The content of galactose-1-phosphate in the supernatant was assayed by HPLC with an H^+ exchange column (Merck Polysher OA KC, Germany), using

25 mM H_2SO_4 as the mobile phase with isocratic elution and a refractive index detector. The column temperature was 30°C. The amount of galactose-1-phosphate produced was detected at 410 nm. Within the time and conditions of the assay, the formation of galactose-1-phosphate was linear with respect to time [27]. Galactokinase activity was defined as: 1 U is equivalent to 1 mM o-nitrophenol produced per min [28].

RT-qPCR Analysis

For RNA extraction, an appropriate amount of mycelia was collected by suction filtration after being cultured for 24, 60, or 96 h. Then it was ground in liquid nitrogen. A certain amount of Trizol reagent (Sangon, Shanghai, China) was added, and total RNA was isolated. Synthesis of cDNA from the total extracted RNA was conducted using the HiFiScript gDNA Removal cDNA Synthesis Kit (CWBiotech, China), according to the manufacturer's instructions. The RT-qPCR reaction was carried out in triplicate in a CFX96 Touch Real-time PCR Detection system (BioRad, USA). The PCR reaction was set up according to the manufacturer's instructions. The relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method, using *sar1* expression as the reference gene for normalization [29]. The primers for gene amplification are listed in Table S1.

### Whole-Genome Sequencing Analysis and Transcriptional Analysis

Whole-genome shotgun sequencing of the hyperproducing mutant was conducted on the Illumina Genome Analyzer (HiSeq 2500) by Sangon Biotech, Shanghai, China. The general process was previously described by Ivanova *et al*. [30]. Two independent samples were analyzed, and the genome sequences were uploaded to SRA with access numbers: SRP124905.

Results

Screening of Hyperproducing Mutants from *T. reesei* RUT-C30

Since the whole-genome sequencing of *T. reesei* was completed in 2008, there have been more than 20 genes that have been identified as being directly associated with the secretion of cellulases [6]. Although many genetic engineering methods have already been successfully implemented in the study of *T. reesei*, the progress is still slow. In this study, ARTP-LiCl compound mutagenesis was applied to acquire mutants with high cellulase activity. As the lethality rate of the ARTP mutation system was dose-dependent, the relationship between various plasma treatment times and the lethality rate of *T. reesei* RUT-C30 was investigated (Fig. 1). The lethality rate was calculated by a previously reported method [31]. As shown in Fig. 1A, the treatment times with successful mutagenesis were 80, 140, 200, 220, 240, and 260 sec. The treatment time of 240 sec was selected

to obtain hyperproducing mutants from *T. reesei* RUT-C30 because this was the time point at which mutants with higher cellulase activity were found. Congo red can form a red complex with certain polysaccharides, such as cellulose, CMC, and hemicellulose, but it cannot react with cellobiose, glucose, or polysaccharide hydrolyzate. Therefore, the Congo red method produces unstained circular zones around the colonies, and it strongly contrasts with the red background of undigested substrate after treatment with NaCl solution [22]. In this study, the screening strategy was based on the size of the hydrolysis halos produced by colonies. The selected mutants were cultured in fermentation medium to determine the FPU activity. The mutant colony with the highest FPU was selected for the next round of mutagenesis. After 5 rounds of mutagenesis, the hyperproducing mutant *T. reesei* JNDY-13 was obtained.
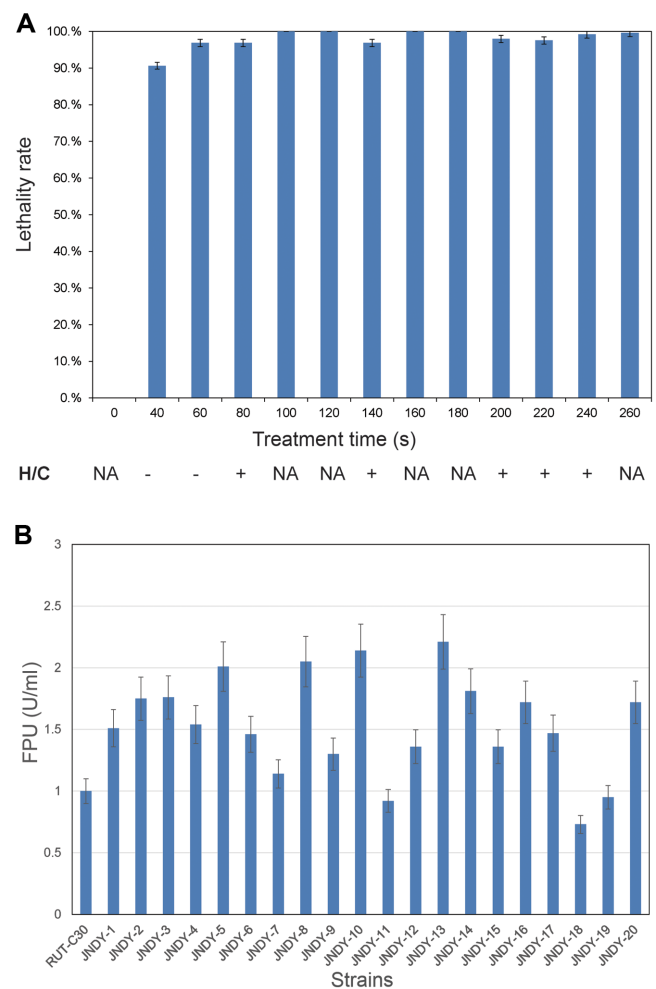
Fig. 1. *T. reesei* mutants created by the ARTP mutation system. (**A**) Lethality rate and (**B**) FPU activity of mutants.
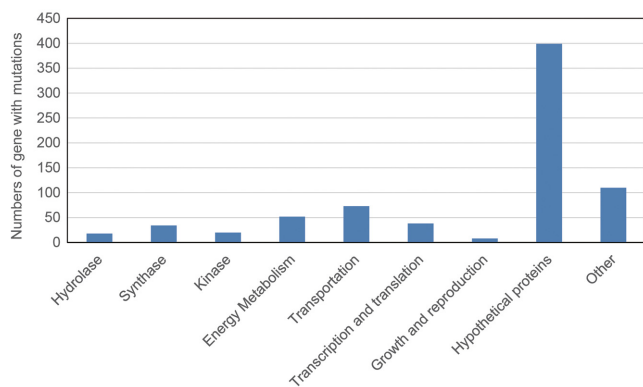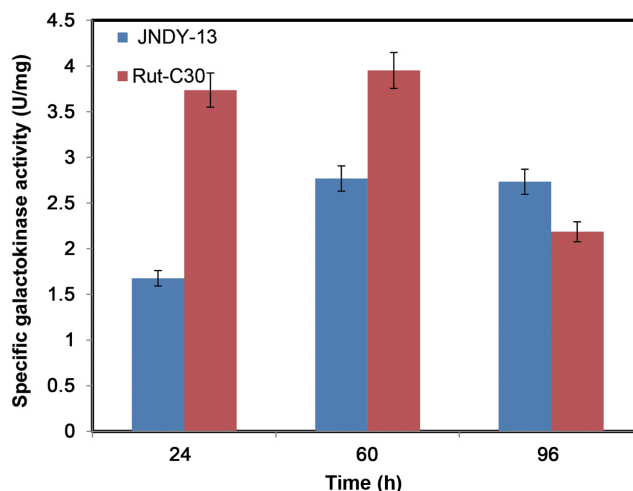
Fig. 2. Categories of the mutant genes of *T. reesei* JNDY-13.

Fig. 3. The specific galactokinase activity of *T. reesei* Rut-C30 and *T. reesei* JNDY-13.

(Fig. 1B). The filter paper activity of *T. reesei* JNDY-13 was 2.21 FPU/ml, which was 2.21 times higher than that of *T. reesei* RUT-C30.

Whole-Genome Sequencing of JNDY-13

High-throughput genome sequencing of the hyperproducing mutant JNDY-13 was executed by the Illumina Genome Analyzer (HiSeq 2500) to study genetic changes. Sequence reads generated by the Illumina system were aligned to a reference genome of *T. reesei* Rut-C30 [6], using the MOM algorithm [32]. For JNDY-13, there were 27,934,787 (94.69% of total) single-end reads that were mapped by Illumina sequencing. All of the sequencing data generated were analyzed for point mutations (SNPs) and other mutational events [12]. There were 752 mutations (Table S2) that were identified in JNDY-13. Among these, 18 were hydrolases, 34 were synthases, 20 were kinases, 52 were related to energy metabolism, 73 were related to transportation, 38 were related to transcription and translation, 8 were related to growth, 399 were hypothetical proteins, and 110 were related to other functions (Fig. 2). Also, 105 out of the 653 SNPs were confirmed to be point mutations, 336 mutations were deletions, and 165 were insertions. Moreover, 99 mutations were insertions and duplications. There was one important mutation that occurred in the galactokinase gene. The "G" at 2015901 of the galactokinase gene was replaced by ""GGGTTAAAAAGCGACTCAC". Galactokinase has been found to be directly related to the cellulase activity of *T. reesei* [33]. *Gal1*-deleted strains down-regulate the induction of cellulase gene expression caused by lactose. Thus, galactokinase could have a significant effect on cellulase production in *T. reesei*. Therefore, an enzymatic assay of this protein was performed. As shown in Fig. 3, the activity of galactokinase in the hyperproducing mutant

was lower than in the parent strain (*T. reesei* Rut-C30) at 24 and 60 h, but it was slightly higher at 96 h.

Transcriptional Analysis of Key Genes Associated with the Secretion of Cellulases

In *T. reesei*, there were five endoglucanases (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), two exo-acting cellobiohydrolases (Cel7A and Cel6A), and two characterized β-glucosidases (Cel1A and Cel3A) that contributed to the total cellulase activity [34, 35]. Moreover, the *T. reesei* genome also contained sixteen hemicellulases, including four GH 95, one GH 10, one GH 74, four GH 11, one GH 67, one GH 62, two GH 54, and two GH 43 [35]. Cell samples were

Table 1. RT-qPCR results of JNDY-13 compared to RutC-30.

Genes	24 h	60 h	96 h	Protein
cel12A	5.553	0.036	0.134	Endoglucanase
cel3C	14.689	19.382	0.026	β-Glucosidase
cel3A	3.775	1.062	0.166	β-Glucosidase
cel61A	0.628	3.638	0.162	Endoglucanase
cel5A	0.666	1.982	0.309	Endoglucanase
cel7A	2.996	0.984	0.025	Cellobiohydrolases
cel6A	0.185	0.0002	0.714	Cellobiohydrolases
cel3B	15.834	3.0244	0.122	β-Glucosidase
cel7B	0.426	44.838	0.189	Endoglucanase
xyn1	28.707	1.145	0.019	Xylanase
xyn2	0.006	1.573	0.298	Xylanase
gal1	0.155	24.391	1.401	Galactokinase

The unit of numbers is fold.

harvested at 24, 60, and 96 h, and they underwent RT-qPCR analysis (Table 1). The transcription of the *gal1* gene was significantly increased at 60 h, when compared to Rut-C30 (24.391). Additionally, 8 of the 11 cellulase and hemicellulase genes of JNDY-13 were up-regulated at 60 h. Thus, the increased transcription of the mutant *gal1* gene could be related to the transcription of the cellulase genes. The up-regulation of the cellulase genes might be the reason why JNDY-13 had considerably higher cellulase activity compared to Rut-C30.

Fermentation Optimization

The key factors affecting cellulase production include carbon and nitrogen sources, fermentation temperature, pH, and dissolved oxygen levels in the fermentation medium. As a consequence of the complexity of the process in which cellulases act as both the product and the catalyst for the decomposition of cellulose, the appropriate regulation of the fermentation conditions, such as pH and temperature, is important for the optimal performance of the production process [36].

The medium optimization experiments were designed based on the Taguchi orthogonal method [37, 38]. According to the results, the optimal composition of the fermentation medium for JNYD-13 was determined to be as follows: 10 g lactose, 10 g avicel, 12 g corn steep liquor, 1.5 g $(NH_4)_2SO_4$,

Table 2. Intuitive analysis.

Factor	Lactose	Avicel	Corn steep liquor	$(NH_4)_2SO_4$	$CaCl_2$	$MgSO_4$	FPU (IU/ml)
Group 1	1	1	1	1	1	1	1.70
Group 2	1	2	2	2	2	2	2.02
Group 3	1	3	3	3	3	3	2.35
Group 4	1	4	4	4	4	4	2.12
Group 5	1	5	5	5	5	5	1.60
Group 6	2	1	2	3	4	5	1.76
Group 7	2	2	3	4	5	1	1.81
Group 8	2	3	4	5	1	2	1.66
Group 9	2	4	5	1	2	3	1.39
Group 10	2	5	1	2	3	4	1.12
Group 11	3	1	3	5	2	4	1.60
Group 12	3	2	4	1	3	5	1.76
Group 13	3	3	5	2	4	1	1.82
Group 14	3	4	1	3	5	2	1.61
Group 15	3	5	2	4	1	3	1.62
Group 16	4	1	4	2	5	3	1.82
Group 17	4	2	5	3	1	4	1.93
Group 18	4	3	1	4	2	5	0.25
Group 19	4	4	2	5	3	1	0.40
Group 20	4	5	3	1	4	3	2.01
Group 21	5	1	5	4	3	2	0.95
Group 22	5	2	1	5	4	3	0.22
Group 23	5	3	2	1	5	4	2.06
Group 24	5	4	3	2	1	5	1.89
Group 25	5	5	4	3	2	1	1.71
Mean 1	1.957	1.568	0.979	1.785	1.761	1.488	
Mean 2	1.547	1.546	1.570	1.734	1.395	1.560	
Mean 3	1.684	1.626	1.931	1.872	1.316	1.567	
Mean 4	1.282	1.484	1.815	1.348	1.583	1.766	
Mean 5	1.366	1.611	1.539	1.096	1.780	1.452	
Range	0.675	0.142	0.952	0.776	0.464	0.314	

Effects of Medium Components on Enzyme Production: Corn steep liquor > $(NH_4)_2SO_4$ > Lactose > $CaCl_2$ > $MgSO_4$ > Avicel

**Table 3.** Orthogonal experiment to find the optimal parameters for cellulase fermentation.

	Temperature (℃)	Agitation (rpm)	Aeration (vvm)	FPU (U/ml)
Group 1	28	400	1	4.74
Group 2	28	600	2	4.26
Group 3	30	400	2	1.22
Group 4	30	600	1	5.63

1 g MgSO$_4$, 1 g CaCl$_2$, 2 ml Tween 80, and 1 ml Mandels solution per liter medium (Table 2).

The best temperature, aeration, and agitation rates were investigated by the orthogonal method [37, 38]. The temperature was set at 28℃ or 30℃. The agitation rate was set at 400 or 600 rpm, and aeration rate was set at 1 or 2 vvm in the 5-L bioreactor. As shown in Table 3, the orthogonal analysis results indicate that the order of importance of the parameters affecting cellulase production was: agitation > aeration > temperature. The optimal conditions for cellulase production were: 28℃, 2 vvm, and 400 rpm. The result of the batch fermentation under optimal conditions is shown in Fig. 4. The highest cellulase activity was 4.35 U/ml at 96 h, which was almost twice that of what was observed in the shake flask.

Fed-Batch Fermentation

Consistent with most results, the most suitable temperature for cellulase production was 28℃, and a temperature of 30℃ contributed to their growth. In this study, the growth was noticeably lagged when 28℃ was chosen, and it took much longer to accumulate a certain amount of biomass. Since cellulase production is closely related to the
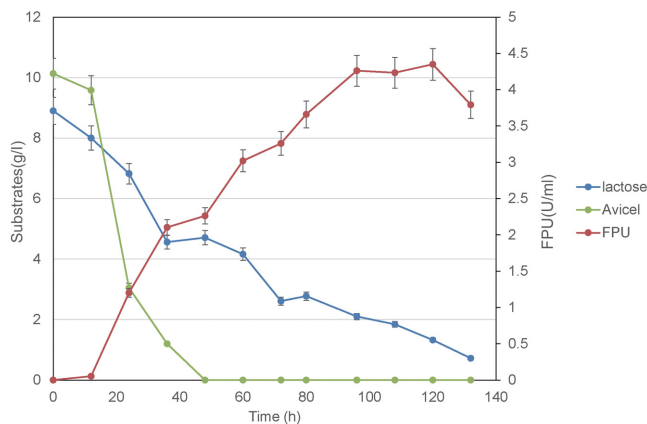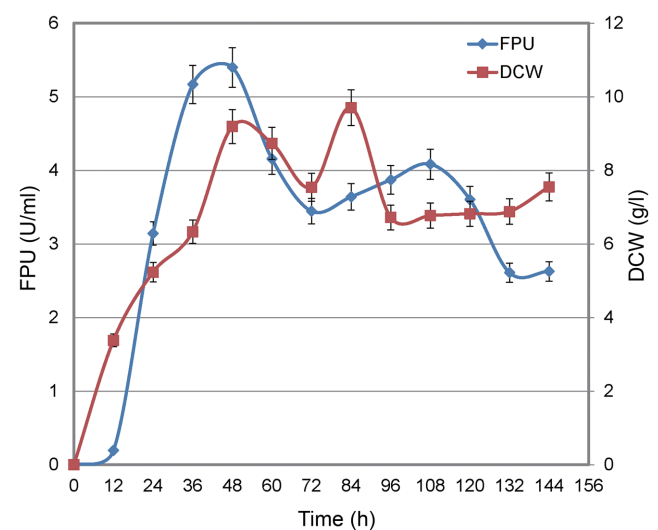

Fig. 4. Cellulase production of *T. reesei* JNDY-13 by batch fermentation.

accumulation of biomass, 30℃ was chosen for a high yield of cellulases. Agitation was set at 400 rpm, and aeration was set at 1 vvm during the batch fermentation [39]. After the cellulose in the fermentation medium was completely degraded, lactose and ammonium sulfate (at a molar ratio of 10:1) were supplemented. The maximum amount of lactose added was less than 50 g/l. The highest cellulase activity was 5.40 U/ml.

Discussion

Despite the continuous efforts of many laboratories, there are no commercially efficient cellulases that have been produced yet [40]. In this study, ARTP, a novel mutagenesis technology, was employed to find a mutant strain of *T. reesei* that could utilize lactose to efficiently produce the cellulase complex. Compared with traditional mutagenesis techniques, ARTP can cause extensive damage to DNA instantly at both room temperature and atmospheric pressure. Its superior characteristics, which include operational safety, lower costs, high mutation rate, and perfect stability of mutant strains, boost its widespread application in the mutation breeding of microorganisms [13–15].

To achieve this goal, a small amount of lactose (3 g/l) was added to selective plates. Colonies that were surrounded with large halos that grew quickly on the selective agar plates were chosen. After 5 rounds of mutation, a hyperproducing mutant, *T. reesei* JNDY-13, that had a cellulase activity of 2.21 U/ml, was obtained.

Fig. 5. Cellulase production of *T. reesei* JNDY-13 by fed-batch fermentation.

In *T. reesei,* which is a hypercellulolytic fungus, the carbon source plays an important role in the production of cellulases. It is well-known that cellobiose, cellobiono-δ-1,5-lactone, lactose, and sophorose could enhance the production of cellulases. However, lactose is the only soluble and economically available carbon source for the production of cellulases [41−43]. Muthuvelayudham *et al.* revealed that the accumulation of cellulases in *T. reesei* QM9414 was increased when a compound of lactose and cellulose was supplemented in the fermentation medium [44]. To find a cost-effective recipe, a mixture of lactose and avicel was chosen as the carbon source, and corn steep liquor and $(NH_4)_2SO_4$ were chosen as nitrogen sources. Additionally, certain metal salts and Tween 80 were also supplemented. Then, the concentrations of the six main components were optimized by the orthogonal method using FPU as the only criterion. After batch fermentation optimization, the highest cellulase activity was 4.35 U/ml. Moreover, with fed-batch fermentation, the highest cellulase activity reached 5.40 U/ml.

Compared to Rut-C30, the whole-genome sequencing of the hyperproducing mutant JNDY-13 identified 752 genetic changes. Among them, the mutation in *gal1*, which encodes galactokinase, was of great importance because it has been reported to be closely related to the production of the cellulases complex. In fungi, lactose is hydrolyzed to galactose and glucose. Then, glucose directly enters the glycolytic pathway, and galactose enters the Leloir pathway [45]. It is subsequently converted to glucose 6-phosphate through multiple enzymatic reactions. The galactokinase of *T. reesei* acts as a regulatory factor in D-galactose metabolism. It is believed to be critical for both the induction of gene *gal7*, which belongs to the Leloir pathway, and the induction of cellulases by lactose [33, 46]. In this study, the increased transcription of the *gal1* gene might be related to increased cellulase production.

Most of the genes related to cellulases complex are regulated in a consistent way [34]. Until now, five transcription factors have been identified as regulatory factors of cellulase genes: the HAP2/3/5 complex, the positive regulators XYR1and ACE2, the repressor ACE1, and the carbon catabolite repressor CRE1 [47]. XYR1 (xylanase regulator 1) is regarded as the key expression activator of cellulase and hemicellulase genes [48]. Though CRE1 is the main transcription factor that mediates carbon catabolite repression (CCR), it is impaired in the mutant strain RUT- C30 [49]. The HAP2/3/5 complex is thought to be essential for the generation of an open chromatin structure, which is necessary for full transcriptional activation [50].

Other types of mutations that were found were changes to genes closely related to membrane transportation. The mutations of genes associated with the transmembrane transportation and the permeability of cells could have a complicated influence on the secretion of the cellulases. Moreover, several mutations that are closely related to transcription and translation levels, energy metabolism, cell growth, and reproduction were identified. These could also affect the secretion of the cellulases complex.

T. reesei is an outstanding producer of extracellular enzymes, and certain industrial strains have the capacity to produce a surprising amount (about 100 g of extracellular protein per liter) [51]. Recently, the FPU titers of the recombinant strain *T. reesei* zxy-2 reached 2.63 U/ml on glucose [52]. When using wheat bran as a substrate, the maximum cellulase production with *T. reesei* NCIM 992 was 2.63 U/ml [53]. The highest FPU of *T. reesei* D-78058 reached 2.60 U/ml with lactose by batch fermentation [54]. In the current study, a cellulase hyperproducing mutant *T. reesei* JNDY-13 had the highest cellulase activity of 5.40 U/ml during fed-batch fermentation. Whole-genome sequencing was applied to study genetic changes in JNDY-13. This may lay the foundation for subsequent screening and provide novel insights into the development of a cellulase hyperproducing strain for industrial applications.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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