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Genome shuffling of *Trichoderma viride* for enhanced cellulase production

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Abstract Genome shuffling is an efficient approach for the rapid engineering of microbial strains with desirable industrial phenotypes. In this study, we used genome shuffling in an attempt to improve the cellulase production of the wild-type strain *Trichoderma viride* TL-124. The initial mutant populations were generated by ultraviolet (UV) irradiation, low-energy ion beam implantation and atmospheric pressure non-equilibrium discharge plasma (APNEDP). The mutants were subjected to recursive protoplast fusion. The cellulase activities of shuffled strains were assayed after solid-state fermentation using wheat straw, an economical raw material, as substrate. The shuffled strain *T. viride* F161, which was selected from among about 2,000 shuffled strains after two rounds of genome shuffling, exhibited a total cellulase activity of 4.17 U g^{-1} dry weight, which was 1.97-fold higher that of wild-type strain *T. viride* TL-124 (2.12 U g^{-1} dry weight). Analysis by random amplification of polymorphic DNA revealed genetic differences between the shuffled strains and the wild-type strain.

Keywords *Trichoderma viride* · Cellulase · Genome shuffling · RAPD

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Introduction

Genome shuffling is an efficient approach for the rapid improvement of microbial phenotypes (Zhang et al. 2002). It involves multiple rounds of protoplast fusion to allow for recombination of the genomes of the starting mutant populations (Zhang et al. 2010). However, the ability to manipulate microbial strains is limited by a lack of knowledge of their genomic sequence; thus, genome shuffling offers a distinct advantage over recombinant DNA techniques in that it allows recombination between uncharacterized organisms (Yu et al. 2008). In addition, strains obtained by genome shuffling can be used in the food industry because they are not considered to be genetically modified organisms (Ahmed 2003). To date, genome shuffling has been successfully used in a wide variety of applications: rapid enhancement of tylosin production in *Streptomyces fradiae* (Zhang et al. 2002); efficient degradation of pentachlorophenol (Patnaik et al. 2002); hydroxycitric acid production in *Streptomyces* sp. (Hida et al. 2007); acid tolerance in *Lactobacillus* (Wang et al. 2007); L-lactic acid production and glucose tolerance in *Lactobacillus rhamnosus* (Yu et al. 2008); thermo-tolerance and ethanol-tolerance; ethanol productivity in industrial yeast (Shi et al. 2009); cellulase production in *Penicillium decumbens* JU-A10 (Cheng et al. 2009); vitamin B12 production by *Propionibacterium shermanii* (Zhang et al. 2010); improved tolerance of *Pichia stipitis* towards hardwood spent sulfite liquor (Bajwa et al. 2010); production of pullulan in *Aureobasidium pullulans* (Kang et al. 2011).

Cellulose, a β-1,4-linked glucose polymer, is considered to be the primary product of photosynthesis and the most abundant renewable carbon source in nature (Jarvis 2003). The bioconversion of cellulosic materials to fermentable

sugars or fuel ethanol by fungal enzyme systems has attracted serious attention from researchers in response to the oil crisis (Szengyel et al. 2000). However, the high cost of cellulase is still the major limiting factor in the hydrolysis of cellulosic materials to fermentable sugars. It is therefore imperative to improve cellulase production to make the process more economically viable (Adsul et al. 2007). To date, many traditional mutagenic strategies have been applied to improve cellulase production (Cherry and Fidantsef 2003; Adsul et al. 2007; Ahamed and Vermette 2008; Chandra et al. 2009; Li et al. 2010), but despite significant efforts, few commercially efficient enzyme complexes have been produced to date. Consequently, the high cost of the enzyme is still the bottleneck in the production of fermentable sugars from cellulosic materials (Adsul et al. 2007).

Novel strains capable of improved cellulase production therefore need to be developed. The aim of the study reported here was to produce a mutant strain of *Trichoderma viride* with enhanced cellulase production by using genome shuffling. We also analyzed the genomes of the shuffled strains by random amplification of polymorphic DNA (RAPD).

Materials and methods

Microorganisms and culture material

The wild-type strain *T. viride* TL-124 was isolated from stacks of wheat straw and maintained on potato dextrose agar (PDA) (Adsul et al. 2007) in test tubes at 28°C. Minimal medium (Tao et al. 2008) was used to cultivate *T. viride*. Cellulose–Congo red medium was used [(in g l^{-1}) KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; Congo red, 0.2; cellulose, 1.88 (Sigma, St. Louis, MO); agar, 20; pH 7.0] for the preliminary selection of mutants.

The wheat straw used to prepare the medium for solid-state fermentation was treated according to the method of Gao et al. (2008). Solid-state fermentation medium was prepared in a 250-ml Erlenmeyer flask containing 10 g of wheat straw, 0.25 g of $(NH_4)_2SO_4$ and 25 ml of distilled water. The medium was autoclaved for 30 min at 121°C.

Mutagenesis

The mutant populations used as the starting strains for genome shuffling were obtained through subtle improvements of the wild-type *T. viride* strain TL-124 by ultraviolet (UV) irradiation, low-energy ion beam implantation and atmospheric pressure non-equilibrium discharge plasma (APNEDP) as described below.

For UV irradiation, protoplasts of *T. viride* TL-124 were prepared as described by Penttila et al. (1987). The protoplast suspension (2 ml of a 10^6 protoplast ml^{-1} suspension) was exposed to UV irradiation according to the method of Rubinder et al. (2002). D-Sorbitol (1 mol l^{-1}) was used as the osmotic stabilizer. Before sporulation, the growing colonies were transferred to PDA slants for further study.

Low-energy ion implantation was performed on UV-mutated *T. viride* according to the method of Su et al. (2006) on the heavy ion implantation facility (patent no. ZL93103361.6, People's Republic of China) of the Chinese Academy of Sciences, Institute of Plasma Physics (ASIPP). Nitrogen ions with an energy of 15 keV were used with an implantation dose of $90 \times 2.6 \times 10^4$ icons s^{-1} cm^{-2}.

For APNEDP mutagenesis, the spores of *T. viride* mutated by low-energy ion implantation were placed 2.0 mm downstream of a plasma torch nozzle exit and treated with pure helium plasma at Pin=180 W and Q_{He}=15.0 slpm. The corresponding gas temperature of the plasma jet was <40°C (Wang et al. 2010).

Genome shuffling

A conidial suspension of *T. viride* TL-124 was prepared by the addition of 5 ml of a 9 g l^{-1} sterile NaCl solution to 7-day-old PDA slant cultures. About 100 μl of the suspension was spread onto a PDA plate and incubated at 28°C. After an incubation of about 18 h, mycelia of the mutated strain were harvested and washed with 0.1 mol l^{-1} phosphate buffer (pH 5.6). Protoplasts were prepared according to the method of Penttila et al. (1987).

Equal numbers of protoplasts from different populations were first mixed and the mixture then divided equally into two parts. One part was inactivated under UV light for 10 min, and the other was heat-treated at 60°C for 5 min (Hopwood and Wright 1979). The two inactivated protoplast samples were mixed at a ratio of 1:1, collected by centrifugation and resuspended in 50 μl of STC buffer (1 mol l^{-1} D-sorbitol, 10 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} $CaCl_2$, pH 7.5). Nine volumes of 60% PEG 6000 in STC buffer were added to the resuspended protoplast mixture, which was then incubated for 6 min at room temperature. STC buffer was added to produce a final total volume of 5 ml, and the fused protoplasts were collected by centrifugation, washed with STC buffer and resuspended in 100 μl of STC buffer. Serial dilutions were regenerated on HMM plates (MM medium with 1 mol l^{-1} D-sorbitol as an osmotic stabilizer) and incubated for 5–8 days at 28°C. The colonies growing on HMM medium were subcultured on PDA to produce a shuffled pool.

Promising shuffled strains identified after one round of genome shuffling served as the starting strains for the next round of genome shuffling.

Fermentation

For the first round of selection, all shuffled strains were screened on cellulose–Congo red screening medium. The most promising strains were selected based on the diameter of the clear zone surrounding each colony. For the second round of selection, the strains selected during the first round were subjected to solid-state fermentation using the economical raw material wheat straw as a carbon source and 0.5% $(NH_4)_2SO_4$ as a nitrogen source. In this medium, wheat straw was used as the inducer for cellulase.

Enzyme assay

The crude enzyme preparation was prepared according to the method of Latifian et al. (2007) and the protein concentration determined by the Bradford method (Bradford 1976) using bovine serum albumin fraction V (Sigma) as the standard. Filter paper activity (FPA, representing total cellulase), CMCase (endoglucanase), β-glucosidase and cellobiohydrolase (exoglucanase) activities were determined according to the methods of Ghose (1987) with modifications. The released sugar was measured by the dinitrosalicylic acid method (Miller 1959).

Random amplification polymorphic DNA analysis

DNA was extracted from mycelia of *T. viride* cultured for 2 days in liquid medium. The mycelia were filtered through Whatman no. 1 filter paper (Whatman, New York, NY) and washed three times with distilled water. Genomic DNA extraction and random amplified polymorphic DNA (RAPD) analysis were performed as described by Wang et al. (2011) and Chandra et al. (2009), respectively. For the RAPD analysis, the primers shown in Table 1 were used. Amplification was achieved using a thermal cycler (PTC200; Bio-Rad, Hercules, CA) set at the following program: 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, 36°C for 1 min and 72°C for 2 min. The products were separated by electrophoresis in 2.0% agarose gels.

Results

Selection of the starting strains for genome shuffling

In this study, wild-type *T. viride* TL-124 was mutated successively by UV irradiation, low-energy ion beam implantation and APNEDP as described in the Materials and Methods. Mutant selection was based initially on the diameter of the clear zones surrounding the colonies grown on cellulose–Congo red medium (Zaldivar et al. 2001). After each mutagenic treatment, the cellulase activities of a mass of clones that displayed promising relatively larger clear zones were assessed by solid-state fermentation using wheat straw as the inducer. The most promising strains based on the results of the cellulase activity assay were subjected to a subsequent mutagenic treatment. After three mutagenic steps, more than 1,000 mutant colonies were produced, four of which (C4, C10, C31, and C36) were selected as the starting strains for genome shuffling.

Enhancement of cellulase productions by genome shuffling

Genome shuffling was applied to improve cellulase production by the *T. viride* mutants C4, C10, C21, and C36 produced by APNED. After one round of genome shuffling, four shuffled strains (E5, E16, E35, and E68) were selected based on their total cellulase activity (3.23, 3.01, 2.87, 3.14 U g^{-1} dry weight, respectively; Fig. 1a). These strains were then used as the starting strains for a second round of genome shuffling, which resulted in two shuffled strains (F62 and F161), with a total cellulase activity of 4.05 and 4.17 U g^{-1} dry weight, respectively (Fig. 1a). Shuffled strain *T. viride* F161 exhibited the highest total cellulase activity among the shuffled strains

Table 1 Primers designed for random amplified polymorphic DNA analysis

Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)
P01	GTCCTACTCG	P11	AGATGCAGCC	P21	AATCGGGCTG
P02	GTAGACCCGT	P12	AGACGTCCAC	P22	GGCTTCTGTC
P03	CAGGCCCTTC	P13	AATGGCGCAG	P23	ACCGCGAAGG
P04	TGCCGAGCTG	P14	GTCAGGGCAA	P24	GGGCAGCTAC
P05	GTGATCGCAG	P15	AAAGTGCGGG	P25	GTCGCCGTCA
P06	CAGCACCCAC	P16	CACGGCGAGT	P26	GTTTCGCTCC
P07	TTCCGAACCC	P17	CTGTCCAGCA	P27	GTGACATGCC
P08	AGCCAGCGAA	P18	GTGTGCCCCA	P28	GGACCCAACC
P09	AGGTGACCGT	P19	AGGGAACGAG	P29	GAGGCCCTTC
P10	TGGACCGGTG	P20	GAAACGGGTG	P30	GTGAGGCGTC

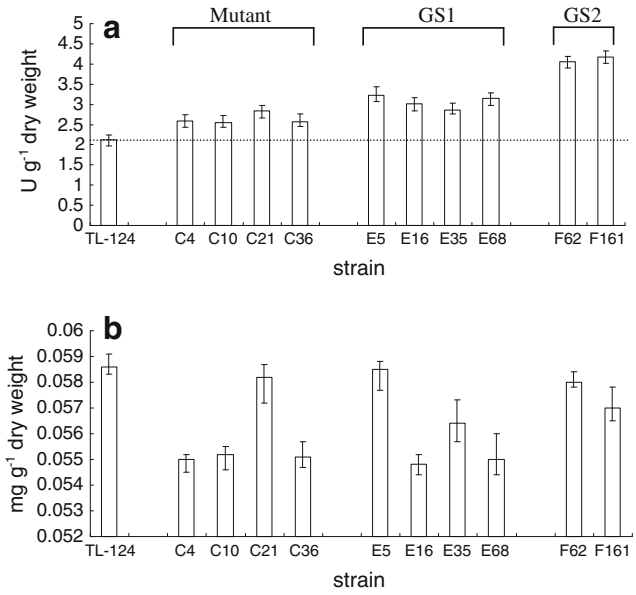

Fig. 1 Improvement in cellulase production due to genome shuffling. Two rounds of genome shuffling were used to improve cellulase production in *Trichoderma viride*. **a** Cellulase production by the shuffled strains [*E5*, *E16*, *E35*, *E68* (*GS1*); *F62*, *F161* (*GS2*)] and wild-type strain *T. viride* TL-124, **b** protein concentration in the shuffled strains and wild-type strain *T. viride* TL-124 determined by the Bradford method (Bradford 1976). *GS1* First round of genome shuffling, *GS2* second round of GS, *C4*, *C10*, *C21*, *C36* mutant strains obtained after mutagenic treatments. The data were produced from a minimum of three replicates. *Error bars* Standard deviation (SD) for each data point

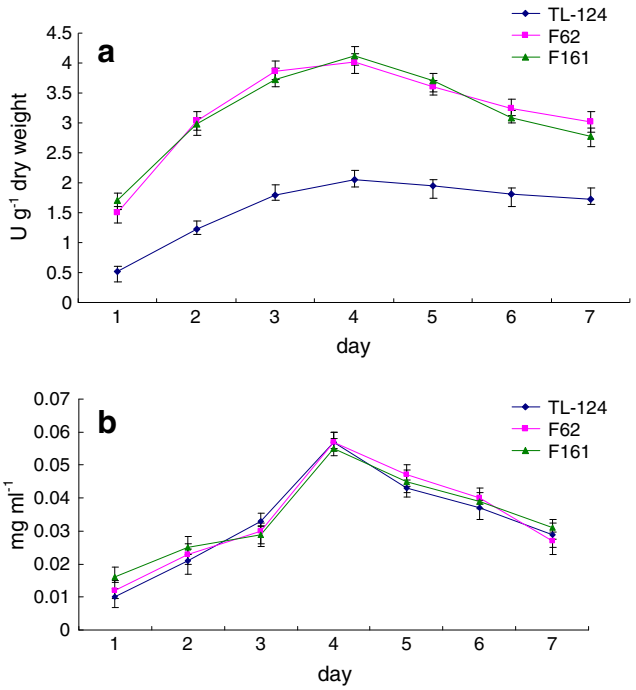

Fig. 2 Time course of the shuffled strains (*F62*, *F161*) and the wild-type strain *T. viride* TL-124. **a** Cellulase production, **b** protein concentrations. The data were from a minimum of three replicates. *Error bars* SD for each data point

after two rounds of genome shuffling, with a total cellulase activity that was 1.46-fold that in the starting strain *T. viride* C21 (2.85 U g^{-1} dry weight), which in turn had the highest activity among the strains obtained after mutagenesis, and 1.97-fold that in the wild-type strain *T. viride* TL-124 (2.12 U g^{-1} dry weight). There was no obvious difference in the protein concentration between the shuffled strains and the wild-type strain (Fig. 1b), indicating that the enhanced cellulase production was not caused by increased extracellular protein secretion.

Cellulase production profiles of the shuffled strains

The time course of cellulase production of the shuffled strains was analyzed using *T. viride* TL-124 as control. The results showed that the wild-type strain and the shuffled strains showed the same trend in the 7-day solid-state fermentation period, except for the level of cellulase production (Fig. 2a) and protein concentration (Fig. 2b). Total cellulase activity and protein concentration in both strains increased continuously during fermentation, reaching a maximum at 4 days and then decreasing with increasing fermentation time. This decrease may have been caused by the autolysis of the mycelia (Fang et al. 2009).

CMCase, cellobiohydrolase and β-glucosidase of the shuffled strains *T. viride* F62 and F161 were assayed together with the wild-type strain after 4 days of solid-state fermentation using wheat straw as substrate. The results showed that F62 and F161 also exhibited higher CMCase (1.70-, 1.68-fold), cellobiohydrolase (2.17-, 2.43-fold) and β-glucosidase (1.35-, 1.53-fold) levels than the wild-type strain *T. viride* TL-124 (Table 2). In a previous assay carried out in our laboratory on the cellulase activity of *T. viride* TL-124 and *Trichoderma reesei* QM9414, which is currently the most commonly used cellulase-producing strain in industrial processess, under solid-state fermentation conditions (Xu et al. 2011), the total cellulase activity of the wild-type strain *T. viride* TL-124 was about 1.24-fold higher than that of *T. reesei* QM9414. These results indicated that the shuffled strains F62 and F161 are promising industrial candidates for the bioconversion progress of cellulosic materials. Moreover, genome shuffling proved to be a successful technique in eukaryotic microorganisms, efficiently improving the production activities of cellulase within a short period of time.

RAPD analysis to identify genomic variation in the course of genome shuffling

Improvements in cellulase activities and alterations in the secretion of cellulase may be affected by several factors.

Table 2 Cellulase activities of the shuffled *Trichoderma viride* and the wild-type strain under solid-state fermentation

Strain	CMCase (U g^{-1} dry weight)	Cellobiohydrolase (U g^{-1} dry weight)	β-glucosidase (U g^{-1} dry weight)	FPA (U g^{-1} dry weight)
T. viride TL-124	44.09±1.96	1.06±0.12	0.56±0.08	2.08±0.13
T. viride F62	75.07±2.15	2.17±0.09	0.76±0.06	3.98±0.12
T. viride F161	74.12±2.34	2.43±0.13	0.86±0.05	4.09±0.15

FPA, Filter paper activity

Data were determined from a minimum of three replicates and are given as the mean ± standard deviation

Genetic variation was expected to occur in the course of genome shuffling.

To confirm genome shuffling, an RAPD polymorphism analysis was carried out using the wild-type *T. viride* TL-124 and the shuffled strains *T. viride* F62 and F161. The DNA banding patterns were similar between the parent and mutant strains except with primer P01 (sequence GTCCTACTCG). Based on the RAPD profiles, shuffled strains *T. viride* F62 and F161 were closer to the parental strain *T. viride* TL-124 (Fig. 3). The results also revealed the presence of two unique bands in shuffled strain *T. viride* F161 that were not present in wild-type *T. viride* TL-124 (Fig. 3). Similarly, one unique band was observed in wild-type *T. viride* TL-124 that was not present in *T. viride* F62. Moreover, shuffled strains *T. viride* F161 and F62 showed different polymorphic bands, as the mechanism of mutation differed between them. Using primer PO1, Chandra et al. (2009) also reported unique DNA banding patterns in parent and mutant strains *T. citrinoviride* EB-104, *T. citrinoviride* EM-563 and *T. citrinoviride* EB-782, possibly indicating that this primer might be adaptive for the differentiation from the mutant cellulase-producing strain. The specific bands obtained in our study show that genetic information was transferred from the parental strains to the shuffled strains by genome shuffling and that the genetic information of the shuffled strains had been changed.

Discussion

The bioconversion of cellulosic materials to fermentable sugars or fuel ethanol by fungal enzyme systems has attracted serious attention from researchers in response to the oil crisis (Szengyel et al. 2000). This has led to cellulolytic fungi, especially *T. reesei* and *T. viride*, being extensively studied for their abilities to produce extracellular cellulases (Zhang et al. 2006). However, the existing *Trichoderma* cellulase systems have several disadvantages, including low enzymatic yields, low specific activities, end-product inhibition and deficient β-glucosidase production (Adsul et al. 2007). Thus, attempts to use these cellulase systems for the degradation of cellulosic materials have not been successful (Zaldivar et al. 2001).

To date, many traditional mutagenesis strategies, such as nitrosoguanidine mutagenesis and UV-irradiation, have been applied to improve the production of cellulase (Cherry and Fidantsef 2003; Ahamed and Vermette 2008). Despite these concerted efforts, however, the high cost of the enzyme is still the bottleneck in the utilization of the raw cellulosic materials (Adsul et al. 2007). It has therefore became imperative to attempt to develop some novel strain with improved cellulase production.

Zhang et al. (2002) reported that genome shuffling was an approach that could be used to enhance the metabolite yield and improve the phenotype of the target strain within

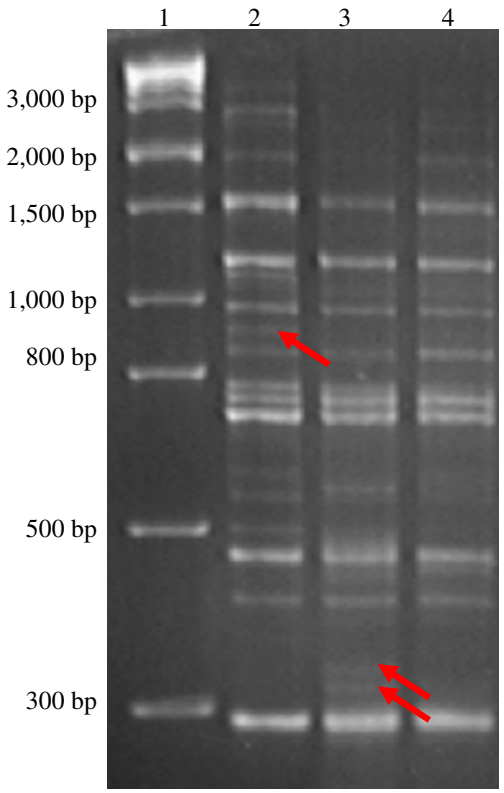

Fig. 3 Random amplified polymorphic DNA analysis of the shuffled and wild-type strains. *Lanes*: *1* 1-kb Plus DNA marker, *2* PCR product from the genome of *T. viride* TL-124 (*arrow*) , *3* PCR product from the genome of *T. viride* F161 (*double arrow*), *4* PCR product from the genome of *T. viride* F62

a short period. In our study, genome shuffling was successfully applied to improve the cellulase-producing strain *T. viride* TL-124. Total cellulase activity of *T. viride* F161, which showed the highest total cellulase activity of all shuffled strains, was about twofold higher than that of the wild-type strain *T. viride* TL-124. This could be further improved by optimization of the cultural conditions or by genetic engineering approaches. Moreover, our comparison of RAPD polymorphism between the parents and shuffled strains revealed that the genotype of the parents and shuffled strains was different, indicating that the genetic information had been transferred from the parental strains to shuffled strains by genome shuffling and that the genetic information of the shuffled strains had been changed. All of the results obtained in our this study clearly demonstrate that the whole genome can be efficiently improved by genome shuffling for the enhancement of the cellulase activities in *T. viride*.

To facilitate the screening and identification of shuffled strains during genome shuffling procedures, the parent strains usually have selective markers. However, any genetic marker that could serve as a powerful selective marker, such as auxotroph protoplasts, would affect the physiology and metabolism of the strains and decrease the cellulase activities of the shuffled strains. Protoplast inactivation is one method that may not affect the activities of cellulase in the offspring. Therefore, we used protoplast inactivation as the selective marker in our study.

Cellulase is a complex enzyme comprised of three components, namely, endo-1, 4-β-glucanase, 1, 4-β-D-cellobiohydrolase and 1, 4-β-glucosidase. The degrading of cellulose to glucose is the synergistically action of these three components. Total cellulase activity, also called filter paper activity, represents the interaction between these three enzymes with the cellulose. Therefore, in this study, total cellulase activity was assayed to determine the ability of the shuffled stains' cellulose to degrade glucose. Martins et al. (2008) revealed that differences in the protein concentration do not allow a direct comparison of the volumetric activities, and that there was a better correspondence when activity values were compared on the basis of protein concentration. Thus, the estimation of protein concentration is vital to the cellulase activity assay. In our study, the Bradford method (Bradford 1976) was adopted.

Solid-state fermentation is an attractive process for economically producing cellulase because of is associated with low capital costs in terms of equipment and low operational costs (Xia and Cen 1999). Nevertheless, the selection of an inexpensive and readily available substrate is essential to lower the cost and optimize the yield of cellulase production (Beg et al. 2000). We therefore investigated the cellulase activities of the shuffled strain *T. viride* F161using solid-state fermentation. Compared with the wild-type strain *T. viride* TL-124, the shuffled strain *T. viride* L161 produced a higher level of cellulase under solid-state fermentation conditions using the economical raw material wheat straw as substrate.

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