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Fungal Strain Improvement for Cellulase Production Using Repeated and Sequential Mutagenesis

Van Hanh Vu, Tuan Anh Pham and Keun Kim*

Department of Bioscience and Biotechnology, The University of Suwon, Hwaseong 445-743, Korea

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A fungal strain producing a high level of cellulase was selected from 320 fungal isolates and identified as *Aspergillus* sp. This strain was further improved for cellulase production by sequential treatments by two repeated rounds of γ-irradiation of Co^{60}, ultraviolet treatment and four repeated rounds of treatment with N-methyl-N'-nitro-N-nitrosoguanidine. The best mutant strain, *Aspergillus* sp. XTG-4, was selected after screening and the activities of carboxymethyl cellulase, filter paper cellulase and β-glucosidase of the cellulase were improved by 2.03-, 3.20-, and 1.80-fold, respectively, when compared to the wild type strain. After being subcultured 19 times, the enzyme production of the mutant *Aspergillus* sp. XTG-4s was stable.

KEYWORDS : *Aspergillus* sp, Cellulase, Sequential repeated mutagenesis, Stability

Cellulose, which is the most abundant renewable resource, is a polysaccharide composed of β-D-glucopyranosyl units joined by 1,4-glycosidic bonds (Gardner and Blackwell, 1974; Kolpak and Blackwell, 1976). Because cellulose can be utilized to produce ethanol, it is a promising alternative energy source for the production of fossil fuels. Cellulose is degraded by cellulases to reducing sugars and fermented by yeast or bacteria to ethanol (Duff and Murray, 1996).

Cellulases are produced by various microorganisms including *Trichoderma* sp., *Chrysosporium* sp., *Fusarium* sp., *Sclerotium* sp., *Phanerochaete* sp., *Aspergillus* sp., *Schizophyllum* sp. and *Bacillus* sp. (Selby and Maitland, 1967; Wood and Phillips, 1969; Toyama and Ogawa, 1975; Sternberg, 1976; Fan *et al*., 1987; Duff and Murray, 1996). Three major types of cellulases, endoglucanases (EC 3.2.1.4), exo-cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21), have been identified to date (Coughlan, 1985; Coughlan and Ljungdahl, 1988).

A reduction in the cost of cellulase production, an improvement in cellulase activity and an increase in sugar yields are all vital to reducing the processing costs of bioethanol from cellulosic substrates (Zhang *et al*., 2006). During ethanol production from lignocellulosics, cellulases play a very important role in the cellulose digestion process and so far the cost of cellulases is very expensive due to the large amounts required for cellulose digestion (Duff and Murray, 1996; Himmel *et al*., 1997; Schell *et al*., 2003; Sun and Cheng, 2002). Therefore, the improvement of microbial strains for the over-production of cellulases has attracted attention in the commercial fermentation process.

*Corresponding author <E-mail : kkim@suwon.ac.kr>

The use of different mutagenic agents for strain improvement was demonstrated by Parekh *et al*. (2000). Additionally, treatment of *F. oxysporum* with ultraviolet (UV) followed by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was used to improve carboxymethyl cellulase (CMCase) production (Kuhad *et al*., 1994). Moreover, Chand *et al*. (2005) used simultaneous treatment with NTG, ethidium bromide and UV or NTG combined with ethidium bromide to create mutant fungi that produced more CMCase and filter paper cellulase (FPase) than wild type fungi.

The purpose of this study was to screen microbial strains for the selection of a strain producing high level of cellulase and to improve the microbial strain further by mutation. In this study, γ-rays of Co^{60}, UV and NTG were repeatedly and sequentially used to mutate the fungal strain to induce the hyper-production of cellulase.

Materials and Methods

Isolation of fungal strains that produce cellulase. A total of 320 fungal strains were isolated from different soils and fruits collected from various sources. One gram samples were then suspended in saline solution (0.85% NaCl) and diluted to 1×10^{-7} g/*ml*. The diluted solution (0.1 *ml*) was then loaded onto a PDA plate (Potato dextrose, 2% agar) containing 0.1% carboxymethyl cellulose (CMC), ampicillin (50 μg/*ml*) and tetracycline (50 μg/*ml*) and then incubated at 30°C for 3~7 days. Next, the plates were stained with 0.1% Congo red dye for 30 min followed by destaining with 1 M NaCl for 15 min. Fungal strains forming a large clearing zone surrounding the colonies were picked up and then streaked onto PDA plates, which were subsequently incubated at 30°C for 7 days. Fifteen fungi (K1~K15) producing higher level of cellu-

lase were selected for further study.

Preparation of seeding culture. Ten grams of wheat bran powder were mixed with 90 *ml* of distilled water in a 250 *ml* Erlenmeyer flask and the pH was then adjusted to 3.5 by 10% HCl. The mixture was then sterilized by autoclaving at 121°C for 20 min, after which it was cooled. One plug (1 × 1 cm^2) of a 7 d-old conidia-mycelia grown on PDA was then added to the flask and incubated at 30°C while shaking at 200 rpm for 1 day.

Solid state fermentation. While both solid and submerged liquid fermentation systems have been used for cellulase production, solid state fermentation (SSF) is generally considered more appropriate for the production of cellulase (Chandra *et al.*, 2008). In this study, an Erlenmeyer flask (500 *ml*) containing 20 g of wheat bran with 33% moisture was plugged with cotton and sterilized for 20 min at 121°C. After cooling, 10% of a 1 d-old liquid fungal seeding culture was used to inoculate the wheat bran, which was subsequently cultured at 30°C for three days.

Preparation of crude enzyme. After 3 days of SSF, the mouldy wheat-bran was mixed with distilled water at a ratio of 1 : 100 (w/v). The mixture was then shaken in an orbital shaker at 200 rpm and 30°C for 60 min. Finally, the supernatant was obtained after centrifugation at 7000 rpm for 10 min for use as a crude enzyme.

Enzyme assay. The activities of CMCase, FPase and β-glucosidase were determined using the method described by Grajek (1987). The CMCase (endo-1,4-β-D-glucanase) assay was conducted in a total reaction mixture of 1 *ml* containing 0.5 *ml* of diluted enzyme and 0.5 *ml* of 1% (w/v) CMC solution in acetate buffer (50 mM, pH 5). The FPase was assayed by incubating 1 *ml* of diluted enzyme solution with acetate buffer (50 mM, pH 5) containing Whatman No. 1 filter paper (50 mg). The reaction mixture for CMCase or FPase was incubated at 50°C for 30 min and the released reducing sugars were then determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The β-glucosidase (β-D-glucoside, glucohydrolase) activity was estimated using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. An assay mixture (1 *ml*) consisting of 0.9 *ml* of *p*NPG (1 mM) and 0.1 *ml* of diluted enzyme was incubated at 50°C for 30 min. The *p*-nitrophenol that was liberated was measured at 420 nm after developing the color with 2 *ml* of sodium carbonate (2 M).

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or *p*-nitrophenol from the appropriate substrates per min under the assay conditions.

Strain improvement by mutations. Of the 15 isolates evaluated, K10 was found to be the most potent fungal strain for cellulase production; therefore, this strain was used for strain improvement by mutation. Two methods of mutation were employed for strain improvement.

Method I: The spores of fungal strain K10 (10^8 spores/*ml*) were harvested from 6 d-old spores grown on PDA plates and then exposed to different doses (0.5 to 2.5 KGy, interval 0.5 KGy) of γ-rays of Co^{60}. The survivors were grown by spreading 0.1 *ml* of the treated spores onto a PDA plate containing 0.1% CMC and subsequent incubation at 30°C for 4~7 days. Based upon the clearing zones surrounding the colonies, γ-ray mutant R-20 was selected and again treated with 2 KGy of γ-rays. According to Kuhad *et al.* (1994) and our modifications, the best γ-ray mutant, R20-2, was sequentially exposed to UV irradiation at 30 W and a distance of 50 cm for 5~60 min, while the spores were removed for testing at 5 min intervals. The best mutant strain, RV-10, was again sequentially treated with four cycles of NTG solution (100 μg/*ml* in 0.2 M citrate buffer solution, pH 5) for 10~60 min, with the spores being taken out for testing at 10 min intervals. The mutant spores were then washed four times with saline solution and spread onto a PDA plate containing 0.1% CMC as described above. After treatment with vari-

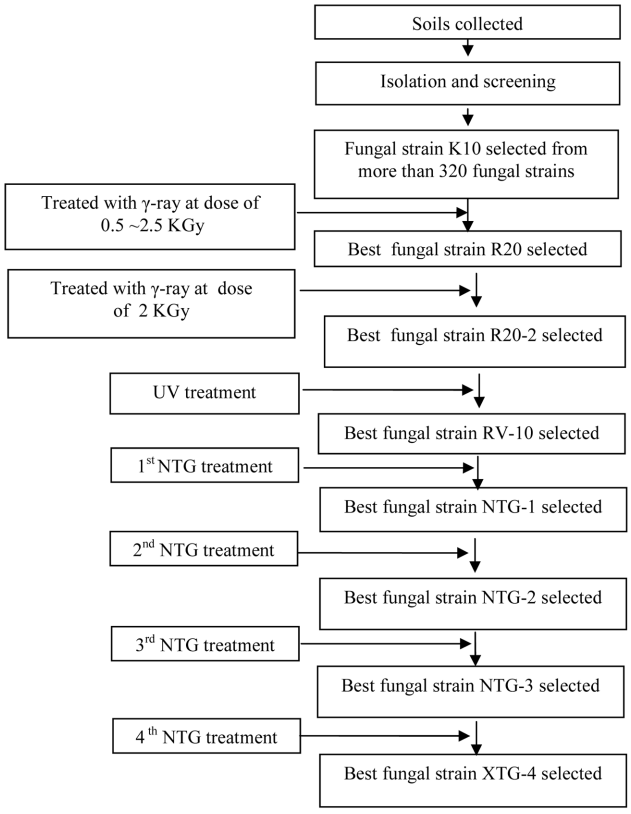

Fig. 1. Sequential mutagenesis for the improvement of cellulase production (Method I). NTG, N-methyl-N'-nitro-N-nitrosoguanidine; UV, ultraviolet.

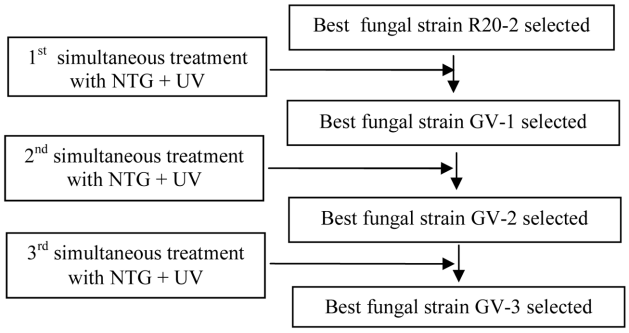

Fig. 2. Sequential mutagenesis with simultaneous treatment of NTG and UV for improvement of cellulase production (Method II). The R20-2 mutant was obtained after the first mutagenic treatment in Method I. NTG, N-methyl-N'-nitro-N-nitrosoguanidine; UV, ultraviolet.

ous mutagenic agents, many mutant strains were screened and tested for the activities of CMCase, FPase and β-glucosidase. Finally, the best mutant strain, XTG-4, which produced a high level of CMCase, FPase and β-glucosidase, was selected for further study (Fig. 1).

Method II: Selected fungal strain R20-2 obtained after the first mutation treatment of Method I (Fig. 1) was further mutated by Method II (Fig. 2). Strain R20-2 was simultaneously treated with NTG (100 $\mu g/ml$) and UV at 30 W and a distance of 50 cm for 5~60 min, with spores being removed at 5 min intervals for testing. The best mutant GV-1 selected was again simultaneously treated with NTG and UV.

Identification of a fungal strain. The DNA of the fungal strains were isolated and purified using a Winzard® Genomic DNA purification Kit (Promega, Madison, WI, USA). The internal transcribed spacers (ITS) of the nuclear rDNA was identified according to the method described by Henry *et al*. (2000) and White *et al.* (1990). Next, polymerase chain reaction (PCR) was conducted using the ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White *et al*., 1990). Specifically, 5 μl of sample containing about 5 ng of DNA was added to the PCR master mixture, which consisted of 5 μl of 10X PCR buffer, 4 μl of dNTP mixture (0.1 mM each dNTP), 0.8 μl of each primer (40 pmol of each primer), and 2 units of Ex*Taq* DNA polymerase (Takara Biomedicals, Osaka, Japan) and diluted to a final volume of 50 μl with de-ionized distilled water. PCR was conducted using a thermal cycler (Genne Amp®, Applied Biosystem, Foster City, CA, USA) to subject the reaction mix to 30 cycles of the following reaction conditions: denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by final extension for 7 min at 72°C to ensure full extension of the products. The amplified PCR products were then purified and sequenced, after which the sequence was aligned with similar well-known sequences obtained from the National Center for Biotechnology Information (NCBI) database.

Statistical program. Data were analyzed using one and two way analyses of variance (ANOVA) ($\alpha = 0.05$) followed by comparison of the means using the Duncan's multiple range test (SAS Institute, Cary, NC, USA).

Results and Discussion

Screening and selection of native fungal strain. Based on the ratio of diameter between the clearing zone and colony on the screening-medium, the fifteen best isolates (K1 to K15) were selected for enzyme production studies. The profile of the enzyme production on the 3rd day of culture of the fifteen selected fungal strains is shown in Table 1. The activity of CMCase produced by the fungal strains differed significantly ($p < 0.005$), and strain K10 showed higher CMCase activity than the other fungal strains ($p < 0.05$). Moreover, strain K10 exhibited higher activities of FPase and β-glucosidase than the other fungal strains ($p < 0.05$) (data not shown). The CMCase, FPase and β-glucosidase activities of strain K10 were 18.73, 11.05 and 15.24 U/*ml*, respectively. Therefore, strain K10 was subjected to strain improvement by mutation.

Improvement of cellulase production by mutation. The production of cellulases by various mutant strains obtained after different mutagenic treatments is shown in Table 2. The improvement of enzyme production by mutant strains

Table 1. The activities of CMCase[a] produced by various isolated fungal strains

Fungal strain	CMCase activity (U/*ml*)
K1	18.41a[b]
K2	15.53ba
K3	10.29bcd
K4	11.94bc
K5	14.98ba
K6	10.64bcd
K7	9.83bcd
K8	18.43a
K9	11.73bc
K10	18.73a
K11	9.54bcd
K12	11.29bc
K13	8.70cd
K14	5.01d
K15	4.99d
Statistical analysis (p)	< 0.05

[a]CMCase, carboxymethyl cellulase.
[b]Means within the same column with the same letter are not significantly different based on Duncan's Multiple Test.

Table 2. Improvement of the production of CMCase[a] by treatment with various mutagenic agents

Method I			Method II		
Mutagenic treatment	Selected mutant	Enzyme production improved (%)	Mutagenic treatment	Selected mutant	Enzyme production improved (%)
None	K-10	100[b]	None	K-10	100[b]
1st γ-ray	R20	127.5	1st γ-ray	R20	127.5
2nd γ-ray	R20-2	138.0	2nd γ-ray	R20-2	138.0
UV[c]	RV-10	142.5	1st simultaneous treatment with NTG[d] + UV	GV-1	141.6
1st NTG	NTG-1	158.4	2nd simultaneous treatment with NTG + UV	GV-2	135.3
2nd NTG	NTG-2	165.3	3rd simultaneous treatment with NTG + UV	GV-3	128.2
3rd NTG	NTG-3	185.3			
4th NTG	XTG-4	202.7[e]			

[a]CMCase, carboxymethyl cellulase.
[b]The 100% CMCase activity was 18.73 U/*ml*.
[c]UV, ultraviolet.
[d]NTG, N-methyl-N'-nitro-N-nitrosoguanidine.
[e]The 202.7% CMCase activity was 37.86 U/*ml*.

was dependant on the mutagenic method used.

Mutagenic method I. Selected fungal strain K10 was treated with two cycles of γ-irradiation (Fig. 1). The best γ-ray-mutant strain, R20-2, was then treated with UV irradiation. The best mutant strain, RV-10, strain was again sequentially treated with four cycles of NTG. Finally, mutant strain XTG-4 was selected (Fig. 1) because it showed a 2.03 fold increase in CMCase activity (Table 2) as well as 3.20- and 1.80 fold increases in the activities of FPase and β-glucosidase, respectively, when compared with the wild type (data not shown). Mutant strain XTG-4 was subsequently identified as *Aspergillus* sp. based on the result of sequencing the ITS region.

Mutagenic method II. Fungal strain R20-2 obtained after the first mutagenic treatment of method I was simultaneously treated with NTG and UV. After treatment, the best mutant selected, GV-1, was again simultaneously treated with NTG and UV (Fig. 2). The resultant mutant showed a 141.6% increase in CMCase activity when compared to the wild type (Table 2). However, further simultaneous treatment with NTG combined with UV led to a rapid decrease in CMCase activity from 141.6% to 135.3%, which was further decreased to 128.2% after a third round of treatment with NTG combined with UV (Table 2). These findings indicated that the simultaneous treatment of NTG with UV was not suitable for improving cellulase production by the fungal strain. Accordingly, based on the enzyme production by mutants created by mutagenic methods I and II, method I, which employed repeated and sequential treatments of different mutagens, was appropriate for the production of fungi that showed improved cellulase production.

It has been reported that when fungi were grown with mutagens at sublethal concentrations, secretive enzyme production increased (Dhawan *et al*., 2003). In the past, the use of mutagenic agents such as γ-rays, UV and NTG for hyper-production of industrial products has been reported (Chand *et al*., 2005; Kuhad *et al*., 1994; Singh *et al*., 1995), but the use of all of these mutagenic agents in sequential studies such as those conducted in the present study have not been documented to date. *F. oxysporum* was treated with UV and then with NTG, after which the activity of CMCase was increased by 3-fold when compared to the wild type (Kuhad *et al*., 1994). Chand *et al*. (2005) used a mixture of NTG, ethidium bromide and UV or a mixture of NTG and ethidium bromide to mutate the fungal strain and found that the resultant mutant strains showed more CMCase and FPase activity than the wild type strains. Singh *et al*. (1995) evaluated spores of *Fusarium oxysporum* DSM841 after UV or NTG treatment and found that the mutants had xylanase and β-xylosidase activities that were 3 fold higher than that of the wild type. However, the present study evaluated the use of sequential treatment with three mutagenic agents, γ-ray, UV, and NTG, to induce the hyper-production of cellulase. Method I used in this study resulted in greatly improved cellulase production by the best mutant strain. Therefore, this method can be applied to achieve potent fungal mutants capable of producing higher amounts of enzymes.

The stability of the mutant *Aspergillus* sp. XTG-4. The stability of mutant *Aspergillus* sp.XTG-4 for cellulase production was determined by successive subculturing on PDA plates for over seven months. After each subculture, the mutant was tested for its ability to stably produce cellulase by SSF. The mutant maintained the same production yield after being subcultured 19 times, indicating that the mutation is stably heritable. Mutant *Aspergillus* sp. XTG-4 was found to consistently produce high activity

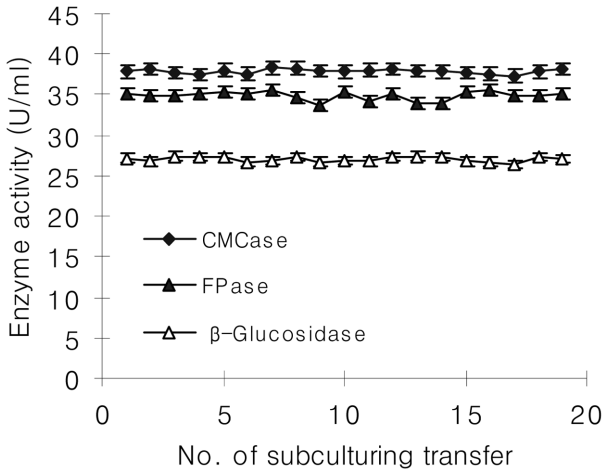

Fig. 3. Stability of the production of CMCase, FPase, and β-glucosidase by mutant *Aspergillus* sp. XTG-4. CMCase, carboxymethyl cellulase; FPase, filter paper cellulase.

CMCase (37.84 U/*ml*), FPase (35.18 U/*ml*) and β-glucosidase (27.12 U/*ml*) on wheat bran over the study period (Fig. 3).

In conclusion, the results of the present study provide valuable information regarding the use of a novel mutation method to obtain mutant microbial strains capable of producing a high level of enzymes.

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