Genetics and Molecular Research

Enhancement of pectinase production by ultraviolet irradiation and diethyl sulfate mutagenesis of a *Fusarium oxysporum* isolate

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ABSTRACT. *Fusarium oxysporum* strain BM-201 was treated with ultraviolet (UV) radiation to obtain a high pectinase-producing strain. Mutant UV-10-41 was obtained and then treated by diethyl sulfate. Next, the mutant UV-diethyl sulfate-43 derived from UV-10-41 was selected as high pectinase-producing strain. Mutant UV-diethyl sulfate-43 was incubated on slant for 10 generations, demonstrating that the pectinase-producing genes were stable. Pectinase activity reached 391.2 U/mL, which is 73.6% higher than that of the original strain.

Key words: *Fusarium oxysporum*; Mutant; Pectinase

INTRODUCTION

Fusarium oxysporum is a soil-borne fungal pathogen that causes more than 100 types of wilting and vascular diseases in plants and produces pectinase (Enciso-Rodríguez et al., 2013; Silva et al., 2013). Pectinases are a large group of enzymes that hydrolyze pectin bonds and are widely applied in different industries. The most common application of pectinase is in the food industry to extract and clarify fruit and beverage juices (Adelakun et al., 2013, Sagu et al., 2014). Pectinases are also used in industrial processes, such as in ramie fiber degumming, oil extraction, coffee and tea fermentation, and industrial wastewater treatment (Esawy et al., 2013, Wu et al., 2013). Pectinases are produced by microbes, plants, and animals, but microorganisms are the most efficient source of the enzyme (Sharma et al., 2013b). Among the different types of microorganisms, pectinase production by filamentous fungi shows the most promise (Sharma et al., 2013a).

The treatment of spores and vegetative mycelium with different mutagens to screen for improved mutants among the surviving progeny has been recognized as an effective means of obtaining strains with improved potency (Khaliq et al., 2009, Romdhane et al., 2013). Strain mutation induction and screening techniques can improve microbes' pectinase yield by using different mutagens such as nitrous acid, diethyl sulfate (DES), and ethyl methyl sulfonate. When fungi are grown with mutagens at sub-lethal concentrations, the rate of enzyme production often increases. Exposure of thermophilic fungal conidia to UV and gamma (γ) irradiation have also been used to obtain mutants that produce higher amounts of pectinase (Siripong et al., 2014). In addition, compound mutations may have synergistic effects and show better results than single mutations. In the present study, we aimed to generate improved strains of *F. oxysporum* using a combination of mutagenesis techniques, including UV irradiation and DES.

MATERIAL AND METHODS

Microorganisms and media

The *F. oxysporum* strain BM-201 was provided by Liwen Laboratory, Department of Biological and Chemical Engineering, Shaoyang University.

The culture of strain BM-201 was maintained on potato dextrose agar slants and Petri-plates. The mutant strain screening medium contained (w/v): 0.5% orange peel powder, 0.2% $(NH_4)_2SO_4$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.06% $MgSO_4$, 0.1% NaCl, and 1.5% agar powder (neutral pH). Mutant strain enzyme production medium contained (w/v): 1.0% orange peel powder, 0.4% $(NH_4)_2SO_4$, 0.06% $MgSO_4$, 0.1% NaCl, 0.1% KH_2PO_4, and 0.001% $FeSO_4 \cdot 7H_2O$ (neutral pH).

Fungal inoculum preparation

The microbial inoculum was prepared by suspending the spores from a potato dextrose agar slant in sterile water. The cells were diluted to 10^6 spores/mL and used for mutation induction and enzyme production.

Mutagenesis by UV irradiation

A 0.2-mL spore suspension was spread onto the isolation plate. Plates were placed

30 cm away from the center of an ultraviolet lamp (ZYT-DDC ultraviolet light, power of 30 W) for 0, 2, 4, 6, 8, 10, 12, or 14 min. After UV irradiation, plates were incubated 2 days in the dark at 30°C to prevent photo-reactivation. A lethal dose curve of the parental strain was plotted, and mutants were isolated from plates showing 75-95% lethal rates. Individual large and white colonies were selected and streaked onto a potato dextrose agar plate. Among the selected strains, the mutant showing the highest pectinase yield was selected for further mutagenesis by DES.

Mutagenesis by DES

One milliliter DES of undiluted solution in a sterile reagent bottle was added to 4 mL anhydrous ethanol. This DES solution was added to 15 mL of the prepared spore suspension. After incubation on an oscillating shaker for 0, 10, 20, 30, 40, 50, 60, and 70 min, 20 mL of sodium thiosulfate was added to terminate the mutagenesis reaction. Next, 0.2 mL processed spore suspension was spread onto the mutant screening plate. Subsequent treatments were the same as those used for UV mutagenesis. Finally, the mutant showing the highest pectinase yield was selected for further analysis.

Enzyme production

Liquid fermentations were cultured in 150-mL Erlenmeyer flasks inoculated with 10^6 spores/mL of the appropriate strain. The flasks were incubated in a rotary shaker at 30°C, 160 rpm. The fermentation broths were centrifuged at 4000 *g* for 20-25 min, and the supernatant was used to analyze pectinolytic activities (Pramod et al., 2014).

Enzyme assay

To draw the standard curve, 10 mg/mL galacturonic acid solution was prepared and then diluted to concentrations of 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL. Each of the 0.5-mL samples was first added to 1.5 mL citric acid-sodium citrate buffer and then 2.0 mL DNS reagent. The samples were boiled for 10 min. Absorbance was measured at 550 nm to draw the standard curve.

To determine enzyme activity, 0.5 mL crude enzyme was added to 1.5 mL sodium pectate and the mixture was incubated at 50°C for 30 min. Next, 2.0 mL DNS was added to the above mixture, and then the mixture was incubated in a boiling water bath for 10 min to stop the reaction. The catalytic activity of the enzyme was determined as the release of 1 mmol galacturonic acid per unit volume, and unit time was defined as 1 unit of enzyme activity. Each test was repeated three times.

Genetic stability of mutant producing enzyme

The mutant obtained by mutagenesis and showing the highest pectinase producing activity was serially passaged for 10 generations in slant culture. For every two generations, pectinase activity was measured to determine the genetic stability of the mutant producing the enzyme.

Relationship between pectinase production and fermentation time before and after mutagenesis

The mutant finally obtained through screening and showing the highest pectinase yield and stable inheritance as well as the original strain were inoculated on a rotary shaker at 30°C, 160 rpm for 7 days. Pectinase activity was measured every 24 h.

Results and discussion

Optimal mutagenesis dose

The death rate of *F.* oxysporum strain BM-201 spores gradually increased with increasing UV exposure time. An exposure time of 14 min was found to be a lethal dose; however, the death rate was 60.5% after 8 min, 86.4% after 10 min, and 97.2% after 12 min (Figure 1). Previous studies reported that a higher rate of positive mutation was observed at death rates of 70-90%, at which mutagenic rates were improved. Therefore, the optimal UV mutagenesis dose was found to be 10 min.

Different DES processing times were used to treat the spore suspension of *F. oxysporum* strain UV-10-41. DES processing for 40 and 60 min resulted in 58.4% and 95.6% death rates of the spores, respectively (Figure 1). The optimal DES mutagenesis treated time was found to be 60 min.

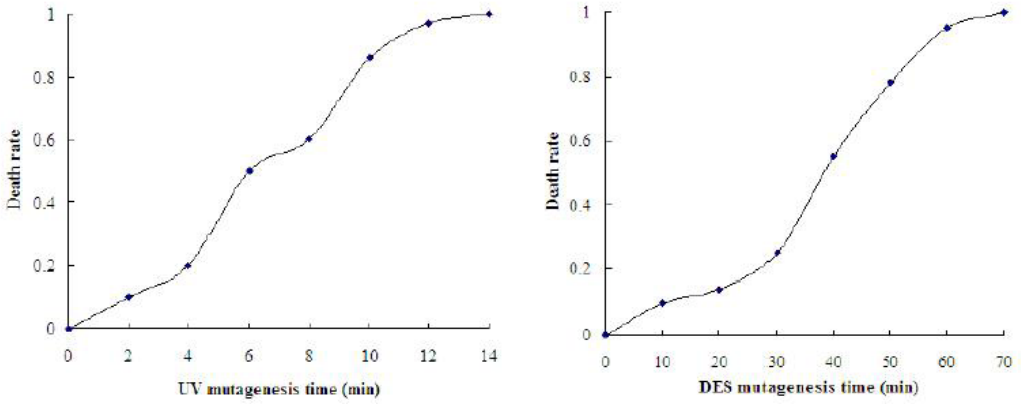

Figure 1. Dose-death curve of UV irradiation (left) and DES (right) treatment.

Standard curve of D-galacturonic acid

The standard curve of D-galacturonic acid is shown in Figure 2, and the concentration of D-galacturonic acid ranged from 0.4 to 2.0 mg/mL. The corresponding absorbance was 0.04-0.70. The equation for the standard curve was y = 0.3884x - 0.1307. The correlation coefficient r = 0.9991 > $r_{0.01(n-2)}$ = 0.977, which was significant. Therefore, the correlation of the equation was high.

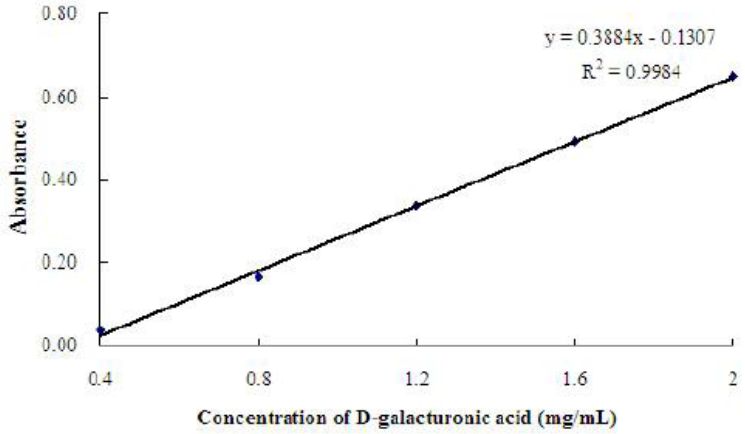

Figure 2. Standard curve of D-galacturonic acid.

Results of UV mutagenesis

A total of 84 mutants were obtained from the original strain BM-201 spore suspension irradiation by UV light for 10 min. Among these mutants, 23 mutants exhibiting rapid growth and colonies larger than the parent stain were selected to measure pectinase production (Table 1). The mutant UV-10-41 showed the highest enzyme activity of 310.5 U/mL, which was improved by 37.8% compared to the original strain. However, using *Aspergillus niger* CICC41254 as the original strain in ultraviolet mutagenesis, a previous study obtained mutant strains through solid state fermentation with pectinase activity improved by 54.24% (She et al., 2012). This may because the mutagenic effects in UV solid state fermentation are superior to those in submerged fermentation.

Table 1. Mutagenic results of UV on strain BM-201.

Strains	Yield (U/mL)	Strains	Yield (U/mL)	Strains	Yield (U/mL)
BM-201	225.4 ± 0.8	UV-8-33	233.4 ± 2.5	UV-10-59	215.4 ± 0.7
UV-10-3	256.3 ± 1.2	UV-10-36	221.5 ± 2.6	UV-10-61	224.0 ± 2.3
UV-10-9	258.6 ± 0.7	UV-10-37	213.6 ± 1.1	UV-10-63	235.6 ± 0.8
UV-10-16	210.4 ± 2.1	UV-10-41	310.5 ± 3.2	UV-10-66	212.7 ± 2.8
UV-10-20	214.8 ± 1.5	UV-10-45	287.1 ± 1.4	UV-10-69	236.8 ± 2.4
UV-10-22	198.3 ± 1.3	UV-10-46	244.3 ± 1.3	UV-10-74	246.2 ± 1.1
UV-10-27	241.1 ± 3.1	UV-10-52	216.8 ± 1.8	UV-10-77	231.3 ± 1.7
UV-10-28	250.6 ± 2.9	UV-10-56	209.5 ± 0.6	UV-10-78	226.8 ± 1.2

Results of DES mutagenesis

The spore suspension of mutant UV-10-41 was mutagenized with 1% DES for 10 min, and 79 single mutant colonies were obtained. Of these mutants, 24 gave rise to colonies that were up to 2-fold larger than those of the parent strain. Their pectinase activity are shown in Table 2. Mutant UV-DES-43 produced the highest enzyme activity, reaching 391.2 U/mL, which is 73.6% higher than the original strain.

Table 2. Mutagenic results of DES on strain UV-10-41.

Strains	Yield (U/mL)	Strains	Yield (U/mL)	Strains	Yield (U/mL)
UV-DES-2	347.3 ± 0.9	UV-DES-26	365.2 ± 1.1	UV-DES-41	363.4 ± 2.4
UV-DES-6	350.1 ± 1.6	UV-DES-27	358.2 ± 2.7	UV-DES-43	391.2 ± 1.3
UV-DES-9	317.9 ± 2.2	UV-DES-28	341.6 ± 3.0	UV-DES-45	301.8 ± 2.1
UV-DES-11	299.6 ± 1.3	UV-DES-30	310.9 ± 2.0	UV-DES-48	278.6 ± 1.9
UV-DES-14	234.2 ± 0.8	UV-DES-32	325.7 ± 1.6	UV-DES-51	286.4 ± 1.8
UV-DES-17	361.4 ± 1.4	UV-DES-33	344.8 ± 1.4	UV-DES-53	373.2 ± 2.0
UV-DES-18	275.8 ± 1.6	UV-DES-38	312.4 ± 2.2	UV-DES-54	345.1 ± 0.9
UV-DES-23	289.5 ± 2.8	UV-DES-39	337.5 ± 2.1	UV-DES-57	325.7 ± 1.5

Genetic stability test of pectinase production of mutant strain UV-DES-43

Mutant UV-DES-43 was serially passaged for 10 generations, and pectinase activity was measured (repeat 3 times) every two generations (Table 3). Statistical analysis revealed that the differences in enzyme activity between 0, 2, 4, 6, 8, and 10 generations were not significant ($P > 0.05$). Thus, the mutant UV-10-43 showed highly stable pectinase production.

Table 3. Genetic stability of pectinase production of mutant strain UV-DES-43.

	Generations						P value
	0	2	4	6	8	10	
Yield (U/mL)	391.2 ± 3.7	389.8 ± 4.0	396.0 ± 3.0	395.5 ± 4.7	389.2 ± 5.3	389.3 ± 5.4	0.1297

Relationship between pectinase production and fermentation time before and after mutagenesis

Mutant strain in the medium inoculated with spores of strain BM-201 and UV-DES-43 were incubated at 30°C in a rotary shaker (160 rpm) for 7 days. Pectinase activity was measured every 24 h and results are shown in Figure 3. As time increased, enzyme production by both BM-201 and UV-DES-43 also increased. Pectinase activity in UV-DES-43 reached a peak on the 4th day (391.8 U/mL), while strain BM-201 showed peak production on the 5th day (225.7 U/mL). After reaching a peak, the pectinase activity in both strains showed a small decrease. This may be because the cells began to die, and metabolite and enzyme binding reduced or eliminated enzyme activity.

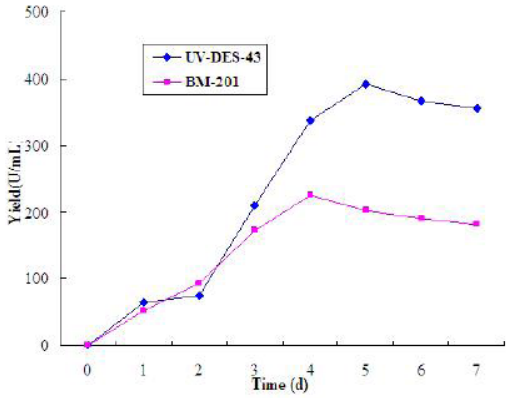

Figure 3. Relationship between enzyme production and fermentation time before and after mutation.

CONCLUSIONS

Fusarium oxysporum strain BM-201 was treated with ultraviolet radiation to develop a high pectinase-producing strain. Mutant UV-10-41 was obtained and then treated with DES. The mutant UV-DES-43 was further analyzed for its high pectinase production. Mutant UV-DES-43 was incubated in slant culture for 10 generations. Pectinase production remains stable over this time period. Pectinase activity reached 391.2 U/mL, which was 73.6% higher than that in the original strain.

Conflicts of interest

The authors declare no conflict of interest.

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