

EFFECT OF GAMMA IRRADIATION ON THE VIABILITY AND CELLULASE PRODUCTION OF SOME FILAMENTOUS FUNGI

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SUMMARY

The motivation of our research is to examine the mutagenic effect of gamma irradiation on cellulase secretion of some filamentous fungi. The spore suspensions of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC were irradiated at dose ranging 0-2500 Gy under gamma Cobalt-60 source at Ha Noi Irradiation Center. The result showed that the survival rate of fungi decreases with the increasing dose. The radiation dose required to kill 90% of the total number of fungal spores (D10) of these strains was about 400 Gy. The viability of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC at 500 Gy were 0.46 % and 0.78%, respectively, while the number of survival spore decreased by 6.5-7.5 Log unit at the dose of 2500 Gy. By screening in PDA medium with the addition of CMC (carboxymethyl cellulose) and Congo red as an indicator of cellulose degradation, hundreds of colonies with higher hydrolysis capacity's value (HC) compared to the initial strain were observed after irradiation. The colonies expressed the highest cellulase hydrolysis capacity with maximum HC value were obtained at dose range of 700-1500 Gy. It is important to notice the 5 potential mutants including 3 mutants of *Aspergillus* (TTG-700, TTG-1000 and TTG-1200) and 2 mutants of *Trichoderma* (VTCC-1000, VTCC-1500) demonstrated the higher CMCase secretion (1.78 – 2.48 times) compared to the wild types. After 5 generations, the enzyme productions of the mutants were fairly stable and there were no differences in growth rates and morphology of each generation. The result of this study is an evidence for using gamma irradiation to improve cellulase production in filamentous fungi.

Keywords: *Aspergillus*, cellulase, gamma irradiation, mutant, *Trichoderma*, spore

INTRODUCTION

Cellulose, a β (1-4) - linked glucose polymer, is considered to be the most abundant renewable carbon resource in the world (Gardner, Blackwell, 1974; Jarvis, 2003). By using cellulase enzyme system, cellulose can be converted to glucose, which is a multi-utility product, in a cheap and biologically propitious process (Gupta *et al.*, 2003). The aerobic decomposition of cellulose is mainly carried out by filamentous fungi. The cellulase complex

secreted by these microorganisms consists of three classes of soluble extracellular cellulase: a 1-4- β -endoglucanase, a 1-4- β -exoglucanase and β -glucosidase, which act synergistically on both the amorphous and crystalline regions of cellulose during the conversion to glucose (Henrissat, 1994; Wang *et al.*, 2011).

The filamentous fungi *Aspergillus* spp. and *Trichoderma* spp. are present in almost all types of soil and many natural surroundings. They have been studied extensively because of their

low-cost culturing and producing efficient cellulase for cellulose degradation. The biomass of these fungi can easily be separated from the culture filtrate, which contains the secreted extracellular enzyme. Thanks to this feature, fungi gained an advantage over the bacteria in the process of industrial cellulase production (Miklaszewska *et al.*, 2016).

Gamma rays are the most energetic forms of ionizing radiation and characterized by their short wavelength, which enables their deep penetration into the matter. Gamma irradiation causes mutations through single-or double-strand breakage of DNA resulting from deletion or structural change, DNA-protein cross links, oxidation, bases, and basic sites (Hoe *et al.*, 2016).

To improve the cellulase production of *Aspergillus* spp. and *Trichoderma* spp., there have been dedicated works focusing on the mutation of these strains by gamma rays. Vu and others (2009) showed that *Aspergillus* sp. was further improved for cellulase production by sequential treatments by two repeated rounds of gamma irradiation of Co-60 (Vu *et al.*, 2009). Gamma irradiation at 2 kGy of *Aspergillus niger* also enhances the production of carboxymethyl cellulase (CMCase) and filter paper cellulase (Fpase) (Mostafa, 2014). Shahbazi and others (2014) reported that *Trichoderma reesei* irradiated at 250 Gy of gamma ray produced a maximum amount of cellulase compared to wild type strains and UV-irradiated mutants. Enzyme assay verified that the gamma irradiated mutants shows approximately 1.21-1.99 fold increases in the activity of each component enzyme of cellulase system in shake flask culture (Shahbazi *et al.*, 2014). The mutants of *Trichoderma reesei* irradiated at 2 kGy of gamma ray secreted 1.8 times as much cellulase as the untreated fungi (Masao *et al.*, 1987).

The purpose of this study is to investigate the effects of gamma ray on the viability of two strains of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC, and using gamma irradiation for

enhancing cellulase production of these strains of filamentous fungi.

MATERIALS AND METHODS

Strains and media

Two rather high cellulase-producing filamentous fungi, *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC, were purchased from Institute of Microbiology and Biotechnology, Vietnam National University, Ha Noi.

Potato destrose agar (PDA) media, 3,5-dinitrosalicylic (DNS) and other chemicals at analytical grade were purchased from Merck, Gemany. Carboxyl methyl cellulose (CMC), Congo red, streptomycin was bought from Sigma.

The medium for shaking flask culture contained: CMC 2 g/L, KH₂PO₄ 4 g/L, (NH₄)₂SO₄ 13.6 g/L, CaCl₂ 0.8 g/L, MgSO₄ 0.6 g/L, pepton 0.1 g/L, yeast extract 0.1 g/L, FeSO₄.H₂O 1 mg/L, MnSO₄.2H₂O 0.32 mg/L, ZnSO₄.7H₂O 0.28 mg/L, CoCl₂.6H₂O 0.4 mg/L, CuSO₄.5H₂O 0.25 mg/L.

Preservation of microorganisms

The wild types of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC were inoculated on a slant of PDA and kept in an incubator at 28°C for 72 hours. Fungi were then stored at 4°C, up to maximum 30 days before sub-culturing.

Preparation of spore suspension

Spore suspensions were prepared using the method described by Darabzadeh and others (2018) (Darabzadeh *et al.*, 2018). The fungi were inoculated on PDA plates and incubated at 28°C for 1 week. The spore suspension of fungi was prepared by adding a sterile saline solution (NaCl 0.9%) to PDA plates and scrapped well. In the next step, the suspensions were distributed in the test tubes and shaken to achieve homogeneity. The final concentration of spore was adjusted to around 10⁸ - 10⁹ CFU/mL.

Irradiation

The tubes of spore suspension were irradiated in duplicate at the same dose rate with the radiation doses ranging from 100 to 2500 Gy under gamma ray ⁶⁰Co source. Actual absorbed doses were measured by Gammachrome YR dosimeters.

Survival assay

The ten-fold serial dilutions of the unirradiated and irradiated spore suspensions were prepared in saline solution, then 0.1 mL of the appropriate diluted suspensions were inoculated on PDA plates (3 plates for 1 dilution), incubated at 28°C for 72 hours for determining the effects of gamma radiation on fungal survival. The number of survival spores (Mi) in 1 mL of the suspension were calculated by the formula:

$$Mi \text{ (CFU/mL)} = Ai \times Di/V$$

Where Ai is the average number of colonies for plate; Di is the appropriate dilution and V is the volume of spore suspension inoculated in plate (mL).

Screening potential high cellulase mutants

The cellulose-degradation ability of colonies of two fungal strains were determined semi-quantitatively by the diffusion method on PDA agar plate containing 1% CMC substrate, 0.02% Congo red as an indicator and streptomycin (50 mg/L) (Miklaszewska *et al.*, 2016).

After irradiation, spore suspensions at different doses were immediately diluted and placed onto prepared PDA/CMC/Congo red medium. The plates were incubated at 28°C for 2 days, followed by a 5-day incubation at 37°C. Hypercellulolytic mutants were selected on the basis of the hydrolysis capacity (HC). The HC was calculated as the ratio of the diameter of cellulose hydrolysis zone marked by Congo red to the diameter of colony growing on a given cellulose medium.

Colonies with a HC value greater than 10%

compared to HC value of wild strain were considered as the potential mutants producing high cellulase.

Enzymatic assay

For this purpose, 2 wild types of fungal strains and the irradiated colonies with the highest HC value selected from the prior stage were cultivated separately onto the shake flask culture. After the incubation at 28°C for 7 days, the cultures were centrifuged at 10,000 rpm for 5 min at 4°C. Supernatants were collected as crude enzyme for enzymatic assay (Miklaszewska *et al.*, 2016; Shahbazi *et al.*, 2014).

Endoglucanase activity (CMCase) was determined using the 3,5-dinitrosalicylic acid (DNS) method. The reaction systems were prepared as follows: 100 µL of crude enzyme (appropriately diluted) mixed with 200 µL of 1% (w/v) CMC. The buffer used for dissolving or resuspending the substrates was 50 mM sodium citrate buffer (pH 4.8). The mixtures were kept at 50°C for 20 min. The reaction was then stopped by adding 0.6 mL of DNS reagent. The mixtures were heated in boiling water for 5 min for color development. Absorbance was read by spectrophotometer at 540 nm (2400 Shimazu UV-Vis). One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugars equivalent to glucose per minute during the reaction.

RESULTS AND DISCUSSION

Effect of gamma radiation on the growth of filamentous fungi

The effect of gamma irradiation on the growth of two filamentous fungi strains was determined through their viability in irradiated spore suspension at dose ranging from 100 to 2500 Gy. Figure 1 expressed the correlation between logarithm of survival spore in CFU/mL with radiation dose. The results showed that the fungal viability was considerably affected by gamma radiation, and the survival spore was decreased with the increase of radiation dose. At

the dose range of 100-1200 Gy, the number of colonies quickly reduced, while these amounts were less variable at doses higher than 1200 Gy. It is obvious that the radiation effect does not show significant differences between *Aspergillus* sp. TTG and *Trichoderma* sp. VTTC.

Assessing the impact of gamma radiation on *A. niger*, Ottenheim and orthers (2015) reported that the D10 (the radiation dose required to kill 90% of the total number of microorganisms) was 400 Gy (Ottenheim *et al.*, 2015) and this value is similar to that estimated for other strains of *Aspergillus* reported in various literature (Blank, Corrigan, 1995; Saleh *et al.*, 1988). In the study on the viability of *Trichoderma viride* with gamma irradiation, Baharvand and orthers (2014) indicated that the number of survival

spores was 9.7% at 400 Gy and no germination was observed at dose of 450 Gy (Baharvad *et al.*, 2014). Laura and orthers (2014) reported that D10 of *Trichoderma viride* was about 450-500 Gy (Laura *et al.*, 2014). The D10 of all two fungal strains in our study was also about 400 Gy. The viability of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC at 500 Gy were 0.46 % and 0.78%, respectively, while the number of survival spore decreased by 6.5-7.5 Log unit at the dose of 2500 Gy. These differences could be attributed to several factors that affect on the survival of irradiated spore such as temperature, density of spore suspension, chemical composition of the medium as well as physiological condition of individual cells and their potential for repairing.

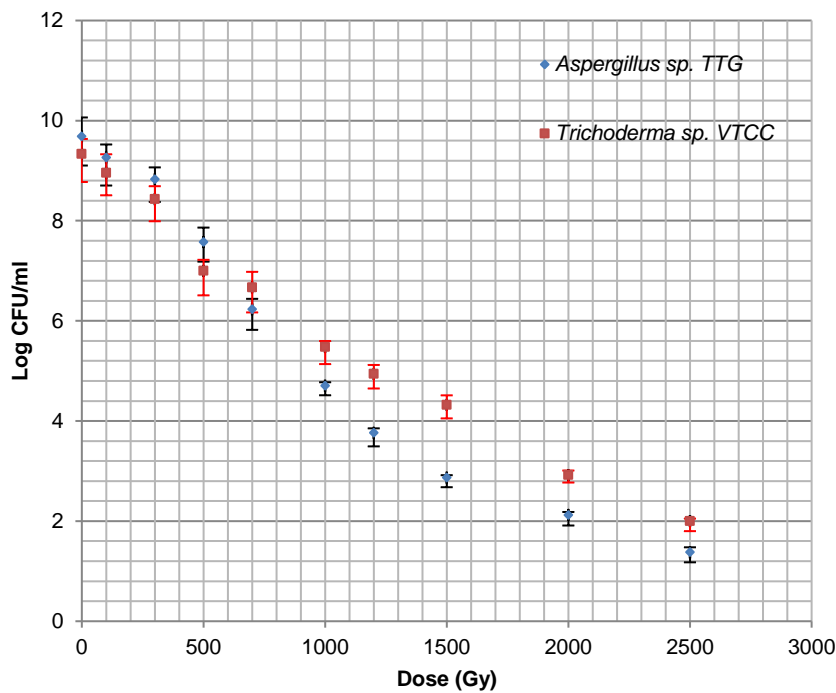


Figure 1. Effects of gamma irradiation on the viability of *Aspergillus* sp. TTG. and *Trichoderma* sp. VTCC.

Enhancement of cellulase production by gamma irradiation treatment

After irradiation, colonies of two fungal

strains with HC value of 10% greater than HC of wild type strains are considered the potential mutants capable of high cellulase producing. The results showed that the colonies with clear zone of

CMC resolution appeared at all irradiation doses on the screening media. However, the number of colonies with a high HC value were different for each dose (Figure 2).

Table 1 shows that the dose range of 700-1500 Gy enabled to create the highest number

of colonies having high HC values, compared to other treatment doses. It is clearly shown in the average HC value of both *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC. Furthermore, the colonies expressed the highest cellulose hydrolysis capacity with maximum HC value were also obtained at this irradiation dose range.

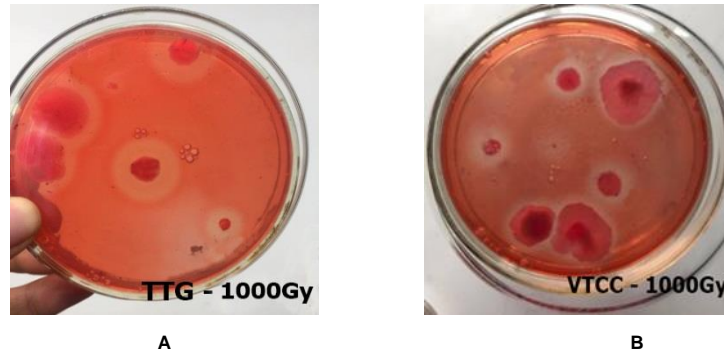


Figure 2. The colonies with clear zone of CMC resolution appeared at dose of 1000 Gy on PDA/CMC/Congo-red screening media (**A**-The colonies of *Aspergillus* sp. TTG and **B**-The colonies of *Trichoderma* sp.VTCC)

Table 1. The cellulose hydrolysis capacity of two fungal strains irradiated at different doses.

| Dose (Gy) | Average HC value | |
|-------------|----------------------------|-----------------------------|
| | <i>Aspergillus</i> sp. TTG | <i>Trichoderma</i> sp. VTCC |
| Wild type | 1.82 | 1.74 |
| 300 | 2.01 ± 0.06 | 1.95 ± 0.10 |
| 500 | 2.06 ± 0.02 | 1.94 ± 0.05 |
| 700 | 2.17 ± 0.08 | 2.05 ± 0.09 |
| 1000 | 2.43 ± 0.12 | 2.21 ± 0.11 |
| 1200 | 2.39 ± 0.10 | 2.17 ± 0.09 |
| 1500 | 2.32 ± 0.04 | 2.06 ± 0.14 |
| 2000 | 2.11 ± 0.09 | 2.01 ± 0.07 |
| 2500 | 2.07 ± 0.10 | 1.95 ± 0.02 |

Cellulase activity

Five potential mutants including 3 mutants of *Aspergillus* sp. TTG (TTG-700, TTG-1000 and TTG-1200) and 2 mutants of *Trichoderma* sp. VTCC (VTCC-1000, VTCC-1500) possessing the highest HC value (HC>2.0 for *Aspergillus* sp. and HC>1,9 for *Trichoderma* sp.) and enzyme activities were selected from hundreds of irradiated colonies. The clear zones of CMC and

CMCase activity of some potential mutants were shown in Figure 3 and Table 2.

Several studies using radiation treatment to create high cellulase-producing mutations in *Aspergillus* spp. and *Trichoderma* spp. also showed the effectiveness of gamma ray (Vu *et al.*, 2009; Mostafa, 2014; Shahbazi *et al.*, 2014; Masao *et al.*, 1987). In this study, some potential mutants were capable of higher CMCase secretion

(2.25-2.37 times for *Aspergillus* and 1.78-2.48 times for *Trichoderma*), compared to the wild type strains were screened at the dose range of 700-1500 Gy.

The stability of these mutants for cellulase production was determined by the successive

subculturing on PDA plates for over 5 months. After 5 generations, CMCase production remained fairly stable with variation coefficients not exceeding 5% (data not shown). In addition, there are no differences in growth rates and morphology of each generation.

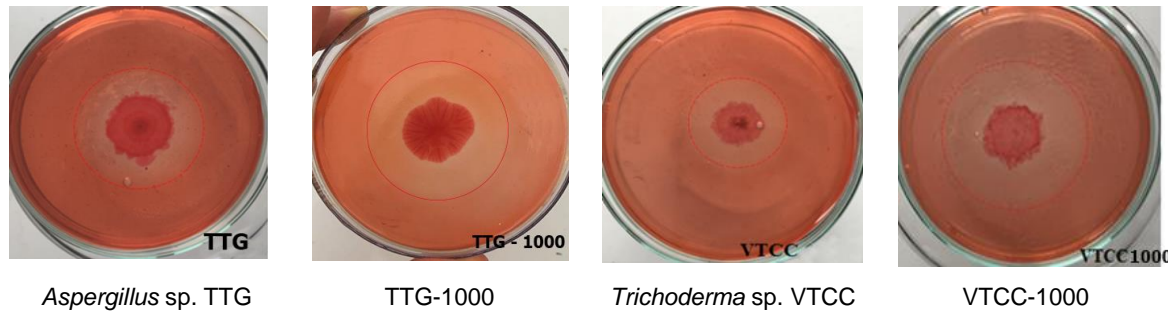


Figure 3. The clear zones of CMC resolution of wild type strains and potential mutants incubated at 28°C for 2 days, followed by a 5-day incubation at 37°C

Table 2. The activities of CMCCase produced by two wild type strains and 5 potential mutants.

| Fungal strain | CMCase activity (U/ml) | Comparison to the wild type strain (times) |
|-----------------------------------|------------------------|--|
| <i>Aspergillus</i> sp. TTG | 1.324 ± 0.04 | - |
| <i>Aspergillus</i> spp. TTG-700 | 2.979 ± 0.12 | 2.25 |
| <i>Aspergillus</i> spp. TTG-1000 | 3.137 ± 0.05 | 2.37 |
| <i>Aspergillus</i> spp. TTG-1200 | 3.071 ± 0.11 | 2.32 |
| <i>Trichoderma</i> sp. VTCC | 1.107 ± 0.04 | - |
| <i>Trichoderma</i> spp. VTCC-1000 | 2.753 ± 0.09 | 2.48 |
| <i>Trichoderma</i> spp. VTCC-1500 | 1.978 ± 0.07 | 1.78 |

CONCLUSION

The viability of *Aspergillus* sp. TTG and *Trichoderma* sp. VTCC was quickly reduced by gamma irradiation. The D10 of all two fungal strains was about 400 Gy and the number of survival spore decreased by 6.5-7.5 Log unit at the dose of 2500 Gy. The 5 potential mutants possessing the highest CMCCase activity (1.78-2.48 times higher compared to wild type) were selected from hundreds of irradiated fungal colonies. The result of the present study is an evidence for using gamma irradiation to improve cellulase production in filamentous fungi.

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ẢNH HƯỞNG CỦA CHIẾU XẠ GAMMA TỚI TỶ LỆ SỐNG SỐT VÀ KHẢ NĂNG SINH CELLULASE CỦA MỘT SỐ CHỦNG NẤM SỢI

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TÓM TẮT

Mục đích của nghiên cứu là bước đầu khảo sát tác dụng gây đột biến sinh cellulase cao ở một số chủng nấm sợi bởi bức xạ gamma. Dung dịch bào tử của *Aspergillus* sp. TTG và *Trichoderma* sp. VTCC được xử lý chiếu xạ ở dải liều 0-2500 Gy trên nguồn gamma Co-60 tại Trung tâm Chiếu xạ Hà Nội. Kết quả cho thấy, tỷ lệ bào tử nấm sống sót giảm theo liều chiếu. Liều gây chết 90% số lượng bào tử nấm (D10) của cả hai chủng này đều khoảng 400 Gy. Số lượng bào tử sống sót của *Aspergillus* sp. TTG và *Trichoderma* sp. VTCC ở liều 500 Gy lần lượt là 0,46% và 0,78% và giảm tới 6,5-7,5 đơn vị Log so với đối chứng ở liều 2500 Gy. Sau chiếu xạ, tiến hành sàng lọc trên môi trường PDA có bổ sung CMC (carboxymethyl cellulose) với chỉ thị Congo đỏ đã thu được hàng trăm khuẩn lạc

có khả năng thủy phân cellulose (HC) lớn hơn chủng ban đầu. Trong đó, các khuẩn lạc thể hiện khả năng thủy phân cellulose cao nhất với giá trị HC tối đa thu được ở khoảng liều 700-1500 Gy. Đặc biệt, 5 thể đột biến tiềm năng bao gồm 3 thể đột biến từ *Aspergillus* (TTG-700, TTG-1000 và TTG-1200) và 2 thể đột biến từ *Trichoderma* (VTCC-1000 và VTCC-1500) có hoạt tính CMCase tăng 1,78-2,48 lần so với chủng gốc. Khả năng sinh CMCase của các thể đột biến được duy trì ổn định ít nhất sau 5 thế hệ liên tiếp, đồng thời không có sự khác biệt về tốc độ sinh trưởng và hình thái ở mỗi thế hệ. Kết quả của nghiên cứu là bằng chứng cho thấy khả năng ứng dụng phương pháp chiếu xạ gamma để tăng cường sản xuất cellulase ở các chủng nấm sợi.

Từ khóa: *Aspergillus*, bào tử, cellulase, chiếu xạ gamma, đột biến, *Trichoderma*