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Screening Streptomycin Resistant Mutations from Gamma Ray Irradiated *Bacillus subtilis* B5 for Selection of Potential Mutants with high Production of Protease

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Abstract: The suspensions of *Bacillus subtilis* B5, a rather high protease production strain, in logarithmic growth phase were irradiated under gamma Cobalt-60 source at Hanoi Irradiation Center. After treatment, the irradiated cells were intermediately cultured in the nutrient agar plates supplemented without and with 20µg/ml streptomycin for screening. The radiation effects on their viability and mutant frequency were studied with radiation dose. The results showed that its survival rate was reduced with the dose as biphasic function. The cells irradiated at dose higher than 1200 Gy do not form colony in the medium containing streptomycin though they could survive in nutrient agar. Therefore, potential streptomycin resistance mutations were collected as survivals from the cells irradiated with radiation dose ranging from 100 to 1000 Gy. Within this dose range, mutation frequency of *Bacillus subtilis* B5 increased with the rising dose. The greatest mutation frequency was determined as 1.61×10^{-3} obtained by irradiation at 1000 Gy, and the smallest as 3.09×10^{-6} at 100 Gy. The enzyme activities of 361 screened colonies from all irradiated samples were investigated in casein agar, and the results revealed 25 colonies having protease activity higher than parent strain.

Keywords: *Bacillus subtilis*, gamma irradiation, streptomycin, survival, mutation frequency, protease.

1. Introduction

Enzymes are natural catalysts synthesized by living organisms to increase the rate of chemical reactions required for life. They have been applied in many various fields from food industry to pharmaceutics and cosmetics. At present, most industrial enzymes are produced by microorganisms because microbial enzymes are more stable than their corresponding plant and animal enzymes.

Moreover, the activity of the microbial enzyme can be easily modulated and their production can scale up. It is estimated that there are about 200 enzymes originated from microorganisms are commercialized [1-3]. Proteases are enzymes that hydrolyze proteins into smaller peptides and free amino acids. And

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microbial proteases have been widely used in food processing, feed production and other industrial applications [4]. *Bacillus* species are the main producers of various enzymes in industrial scale, and *Bacillus subtilis* is frequently used for the production of extracellular proteases [5].

Microbial genome may be modified by physical and chemical mutagenesis such as UV light, γ-ray, antibiotics… in order to increase their level of enzyme production of the wild-type [6]. Among the physical mutagens, ray, one of the radiation emit from the disintegration of ^{60}Co radioisotopes, is the most commonly used mutagen in practice. Gamma radiation induced reactive oxygen species (ROS) that react with DNA, RNA in the irradiated cell, resulting in damages in nucleic acids and nucleotides, leading to mutations or even cell death [6-8]. In some cases, it can create useful mutations at specified loci in genome [9]. Therefore, gamma radiation was considered as an appropriate method to induce microbial mutants for selecting the strain having specific characteristics such as radiation sensitivity, radiation or antibiotic resistance [7].

Recently, ribosome engineering has developed for changing the secondary metabolic function of the wild-type strains and screening potential mutant strains [10]. Streptomycin is an antibiotic, which acts as a potent inhibitor of prokaryotic transcription initiation, can be used to study transcription in bacteria. Ochi K. reported that streptomycin likely attacked to ribosome complexes or RNA polymerase in order to alter the transcription and the translation of microorganism, thus improving enzyme productivity without modifying the genes of the original strain [11]. Several streptomycin resistant mutants of *Bacillus subtilis* have been found to produce increasing amounts (20–30%) of amylase and protease. In addition, *rpo*B mutations created by rifampicin mutagen were effective for the overproduction (1.5- to 2-fold) of these extracellular enzyme [12].

In Vietnam, various strains of useful bacteria have been isolated and exploited for agricultural, industrial and medical applications. However, the mutant strains seem not to be used regardless their advantages in production of primary or secondary products. In recent years, there are some achievements in radiation-induced mutagenesis technique, which have been applied in practice. Unfortunately, most radiation-induced mutations are predominantly point mutations, though the direct action of radiation tends to form larger genetic changes. Combination of radiation and ribosome engineering can reduce screening time, but produce the broad spectrum of mutations with increasing mutation rates. Moreover, the mutagenic effects of radiation are the causes of the development of antibiotic resistance in the exposed colonies [13]. Therefore, gamma radiation and streptomycin has been applied as mutagens in the present study for screening potential streptomycin resistance mutations having improved protease production from *Bacillus subtilis* B5.

2. Materials and methods

A rather high protease-producing strain, *Bacillus subtilis* B5, was kindly supported by Research and Development Biotechnology School, Hanoi University of Science and Technology.

Nutrient Agar (NA) and nutrient Broth (NB) media were purchased from Difco, USA. Streptomycin, CH_3COOH, amido black, casein at analytical grade were bought from Sigma. Other chemicals were bought from Wako, Japan and agar from a domestic company.

Preparation of Bacillus subtilis suspesion in log phase growth. A loopful of *Bacillus subtilis* B5 was taken from the NA plate, put in NB medium, cultured and shaken at 37°C for 24 hours. After that, 0.5 ml of this suspension was dispersed in 50 ml NB in a 100 ml Erlenmeyer flask, and incubated at the same condition to reach log growth phase.

Gamma irradiation. Aliquots of cell culture (about 10 ml) in growth log phase was distributed in the test tubes, then the tubes were irradiated in duplicate at the same dose rate with the radiation doses ranging from 0.1 to 3.0 kGy under gamma ray ^{60}Co source. Actual absorbed dose were measured by Gammachrome YR dosimeters.

Screening potential streptomycin resistant mutations. The ten-fold serial dilutions of the irradiated suspensions were prepared in saline pepton, then 0.1 ml of the diluted suspensions were placed on NA plates, incubated at 37°C for 24 hrs for determining the effects of gamma radiation on bacterial survival. In parallel, 0.1 ml of these cell suspensions were put on the plates of NA containing 20µg/ml of streptomycin for screening potential streptomycin resistance mutations. The same volume of non-irradiation cells was also cultured as negative control.

After incubation period, the survivals were counted as colony forming units (CFU) grown in the medium with and without 20 µg/ml streptomycin from the same irradiated suspension. Mutation frequency was determined as the ratio of the survived colony number in the medium containing streptomycin and those in pure NA medium at various doses.

Isolating extracellular proteases and determining their activities. The potential streptomycin resistance mutations of gamma irradiated *Bacillus subtilis* B5 were used for selecting high protease producing strains. Each colony was inoculated into a 700 µl NB in Eppendorf tubes, incubated at 37°C under shaking condition (120 rpm) for 24 hours. The crude enzyme was obtained by centrifugation of the cell culture at 10000 rpm, at 4°C for 10 min.

Agar was prepared together with 0.1% (w/v) casein and poured in petri dishes. The plates were solidified for 30 min and holes (5 mm diameter) were punched. 30 µl of each crude enzyme was loaded into a corresponding hole. These plates were incubated at 37°C overnight and amido black reagent was flooded to all plates for 20-30 min at room temperature. Finally, the clear distinct zone appeared after dyeing the casein agar plate was observed and photographed. The colony having larger halo zone, namely high enzyme activity were selected as potential protease producing mutation for further study.

3. Results

Effect of gamma radiation on the growth of Bacillus subtilis B5. The growth of the irradiated cells was observed to evaluate the radiation effects on viability of *Bacillus subtilis* B5. After irradiation, all irradiated cell suspensions were immediately inoculated on the same NA plate (5 µl for each), incubated at 37°C for 24 hours. The same amount of non-irradiated suspension was also inoculated on the petri dish for comparison. It was found that there were obvious differences in the colony density between irradiated and non-irradiated bacteria samples (Fig. 1). The number of colonies seems to depend on radiation dose. From the dose higher than 500 Gy the number of colonies quickly reduced, even only 2 conlonies were observed when the sample was irradiated at 3000 Gy.

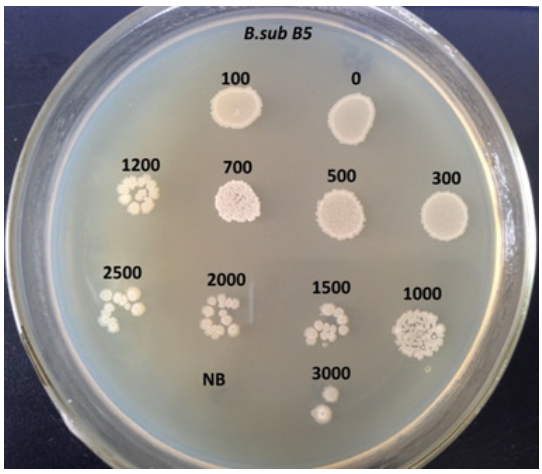

Fig.1. Growth of *Bacillus subtilis* B5 irradiated with various radiation doses compared to non-irradiated one.

It is obviously that bacterial viability was dramatically affected by gamma radiation, and the cell survival was reduced with increase of radiation dose. The effect of radiation on *Bacillus subtilis* B5 was expressed as logarithm of survival cells in CFU/ml with radiation dose (Fig. 2). The results revealed the dose-dependent viability of the irradiated bacteria was biphase curve with reduction of radio-sensitivity of the survivors that irradiated at dose higher than 1200 Gy. It may be due to bacterial aggregation during irradiation, resulting in formation of the larger cell clusters with higher radio-resistant [14].

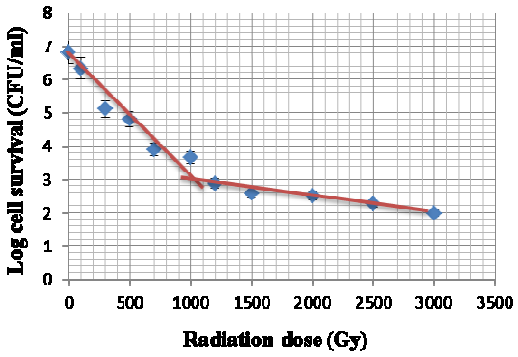

Fig. 2.Effects of gamma irradiation treatment on the viability of *Bacillus subtilis* B5.

Study on the viability of Bacillus spore with gamma radiation, Yoon Ki-Hong et al. [15] indicated that the survival fraction of irradiated spores of *Bacillus* sp.79-23 exponentially decreased in the dose ranging from 0.5 to 5 kGy. At 3 and 5 kGy, the number of survival spores was 5% and 1%, respectively.

In other study, *Bacillus* sp. NMBCC 10023 originally isolated from soil was irradiated with doses of 1-40 kGy. The survival rate of the bacterial culture decreased exponentially with increasing irradiation dosage. Guijun et al. [16] reported that lethal rate of *Bacillus subtilis* NCD-2 increased with irradiation dose, the lethal rate of the bacteria irradiated at 1000 Gy reached 99.50%. Afsharmaesh *et al*. also found the reduction of survival fraction of *Bacillus subtilis* UTB1 by radiation follows a rather linear model [17].

These differences could be attributed to the environmental factors that affect the survival of irradiated cell such as temperature, phase of growth, the nature of gaseous environment, chemical composition of the medium as well as physiological condition of individual cells and their potential for repairing.

Frequency of streptomycin-resistant mutants. One advantage of ribosome engineering is the ability to select the drug-resistant mutants, even at frequencies as low as 10^{-9}-10^{-11} [10]. In this study, streptomycin was used in combination with irradiation treatment to increase the selective pressure, mutation rate, and reduce the screening time for the potential mutations. Resistance to streptomycin is often mediated by mutations within *rrs*, a 16S rRNA gene, or *rps*L, which encodes the ribosomal protein S12- lying on the small region of ribosome [11].

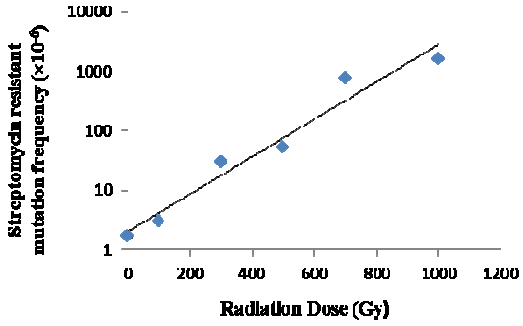

Fig.3. Frequency of streptomycin-resistant mutations of *Bacillus subtilis* B5 exposed to gamma ray at various dose.

Because no streptomycin resistant mutation can be observed in the plate inoculated with the cells irradiated with dose higher than 1200 Gy, only the mutations from the cells irradiated at the dose below 1000 Gy were investigated. Figure 3 showed the frequency of potential streptomycin resistance mutations in *Bacillus subtilis* B5 irradiated with dose of 100-1000 Gy. As one can see that mutation frequency increased with rising radiation dose. The

greatest mutant frequency 1.61×10^{-3} (1 mutation per 621 CFU), was induced by irradiation at 1000 Gy, the smallest one 3.09×10^{-6} (1 per 0.323×10^{6} CFU), was induced by irradiation at 100 Gy. The data also revealed the frequency of spontaneous mutation was about 1.78×10^{-6} in average (1 per 0.56×10^{6} CFU). These results suggested that the resistivity of the irradiated bacteria to streptomycin somewhat improved by radiation treatment.

Protease activities of potential streptomycin resistant mutations. Protease activities of the crude enzymes secreted from the potential streptomycin resistant colonies which grown on the NA containing 20 µg/ml streptomycin of the irradiated *Bacillus subtilis* B5 were determined by well diffusion method. Formation of halo zone around the colony, resulting from casein hydrolysis, is regarded as evidence of proteolytic activity. The protease activity was determined by the size of this clear zone as showed in Figure 4.

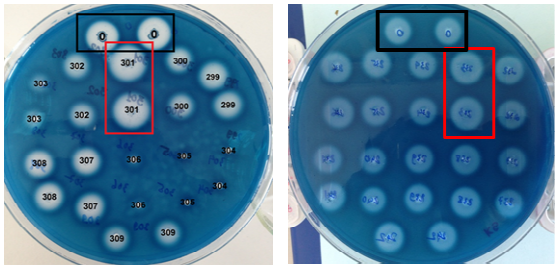

Fig. 4. Casein hydrolyses of the crude proteases secreted by potential streptomycin resistant colonies of the irradiated *Bacillus subtilis* B5 (clear zones in black and red frames were produced by the parent and potential mutant, respectively).

The higher activity of protease the colony had, the larger clear zone was appeared. The diameter of clear zone is therefore proportional to the enzyme concentration. Among the clear zone forming colonies, only larger zone forming colonies were selected as potential mutants for further study. By second screening, 25 potential mutants with higher production of protease were screened from 361 potential streptomycin resistant mutations as indicated in Table 1. However, these potential mutants with improved production of protease should be further studied for searching the stable mutants.

Table 1.Numbers of the potential streptomycin resistant colonies and high protease producing mutants selected from the irradiated *Bacillus subtilis* B5

Radiation dose (Gy)	Number of colonies	Number of colonies with a larger casein degradation zone around enzyme source (CFU)
100	142	10
300	127	10
500	77	5
700	12	0
1000	3	0
Total	361	25

4. Conclusion

The viability of *Bacillus subtilis* B5 was quickly reduced by gamma irradiation. By screening of the irradiated bacteria on the NA containing 20µg/ml streptomycin, we obtained 361 potential streptomycin-resistant mutations, and the mutation frequency increased with rising radiation dose in dose range of 100-1000Gy.

The frequency of spontaneous mutations was averagely 1.78×10^{-6} and the highest mutation frequency was 1.61×10^{-3} observed with the bacteria irradiated at 1000 Gy. The protease activities of the screened colonies were evaluated as their casein hydrolyses, 25 potential mutants with higher production of protease were selected for further studies.

Acknowledgments

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Sàng lọc các đột biến kháng streptomycin từ *Bacillus subtilis* B5 xử lý chiếu xạ tia gamma nhằm chọn các đột biến triển vọng có khả năng sản xuất protease cao

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Tóm tắt: Huyền dịch *Bacillus subtilis* B5, một chủngvi khuẩn sinh protease, ở giai đoạn phát triển theo hàm mũ, được chiếu xạ với nguồn bức xạ gamma Cobalt-60 tại Trung tâm Chiếu xạ Hà Nội. Sau khi xử lý, các tế bào chiếu xạ ngay lập tức được nuôi cấy đồng thời trên đĩa thạch dinh dưỡng thường và đĩa thạch bổ sung 20µg/ml streptomycin để sàng lọc. Ảnh hưởng của bức xạ đến khả năng sống và tần số đột biến của chúng được khảo sát theo liều chiếu. Kết quả chỉ ra rằng tỷ lệ vi khuẩn sống sót giảm theo liều chiếu như hàm hai pha. Không khuẩn lạc nào có thể phát triển từ vi khuẩn chiếu xạ liều trên 1200 Gy được ủ trong môi trường chứa streptomycin dù chúng vẫn có thể mọc trên môi trường không có streptomycin. Vì vậy, các khuẩn lạc phát triển từ vi khuẩn chiếu xạ trong khoảng liều 100-1000Gy đã được xem như các đột biến kháng streptomycin triển vọng. Trong khoảng liều này, tần số đột biến của *Bacillus subtilis* B5 tăng theo liều chiếu. Tần số đột biến cao nhất là $1{,}61\times10^{-3}$ đạt được ở liều chiếu 1000 Gy, và nhỏ nhất là $3{,}09\times10^{-6}$ khi chiếu xạ liều 100 Gy. Hoạt tính protease của 361 khuẩn lạc sàng lọc đã được xác định trong đĩa thạch casein, và kết quả cho thấy có 25 đột biến triển vọng với khả năng sinh protease cao hơn chủng gốc.

Từ khóa: Bacillus subtilis, chiếu xa gamma, streptomycin, tỷ lệ sống sót, tần số đột biến, protease.