Chapter 144
Study on ^{60}Coγ-Ray Irradiation Mutation of *Bacillus subtils* natto

Yufeng Xie and Yingliang Ge

Abstract Soybean meal (SBM) was widely used as raw material in plant protein industry. *Bacillus subtilis* natto was the beneficial bacteria of generating a variety of protease, which could degrade plant protein of SBM, so as to improve the hydrolysis degree (DH) of plant protein. *Bacillus subtilis natto* was commonly treated by ultraviolet ray(UV), Lithium chloride(LiCl), nitrosoguanidine (NTG), diethyl sulphate (DES) at home and abroad, but it had not been reported that bacillus natto was treated by ^{60}Coγ gamma rays (^{60}Coγ). Different dosages of ^{60}Coγ irradiation and 0.85 % Lithium chloride were used as mutagen in treating *Bacillus subtils* natto HRBX0. Result showed that when 400 Gy dosage was used, which led to a 95 % spore death rate with the positive mutation rate 14.5 %, good mutagenic effect occured. DH of defatted SBM in solid-state fermentation of the mutant HRBX6 increased from original 19.5 % to 30.6 %, increased by 56.9 %. It showed a good genetic stability and stably DH of defatted SBM with the experiments of the mutant after continuous cultivation for five generations and obviously exceeded the original strain. It showed that ^{60}Coγ was an effective factor of mutagenic breeding of *Bacillus subtilis* natto.

Keywords ^{60}Coγ · Mutation · DH · *Bacillus subtils* natto

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144.1 Introduction

Bacillus species have been major workhorse industrial microorganisms with roles in applied microbiology, which date back more than a thousand years, since the production of natto by solid-state fermentation of SBM using *Bacillus subtilis* natto is first practiced in Japan. The production of protein for raw materials has exceeded 100 million tons with the development of China in the past 20 years, but at the same time it has become a prominent problem on shortage of resources especially the protein for raw materials [1]. SBM is the major source of protein in animal diets used in China and several other countries, and is widely used as the plant protein industrial raw material [2].

Not high DH is obtained by using the current strains. Much is now known about the biochemistry, physiology, and genetics of *B. subtilis* natto, which facilitates further development and greater exploitation of these organisms in industrial processes. So we need mutants of *B. subtilis* natto. It is reported that *B. subtilis* natto is almost handled by a various of physical or chemical mutagens in domestic and foreign by using UV, LiCl, NTG, DES, and so on [3]. It has not been reported that *B. subtilis* natto is handled by ^{60}Coγ. When SBM is subjected to fermentation with the mutant strain HRBX6, most of the total amino acids increased significantly and only few of them suffer a decrease depending on the type of the fermentation.

Bacillus subtilis natto HRBX0(HRBX0) was used as the original strain. It was processed by different doses of ^{60}Coγ, and then screened the mutant strain. So the HRBX6 with the high DH was obtained. The results showed that the nutritional quality of SBM and its utilization rate of the protein industry was improved.

144.2 Materials and Methods

144.2.1 Original Strain

HRBX0: It was preserved by microbiology laboratory in Harbin institute.

144.2.2 Materials

144.2.2.1 SBM

Defatted SBM containing 48 % protein was purchased from market.

144.2.2.2 Chemicals

PITC (phenylisothiocyanate 99 %) and DL-norleucine were obtained from Sigma. Medium were from "Beijing Land Bridge Technology Co., Ltd". All the other analytical chemicals were from Merck.

144.2.2.3 Medium

Liquid broth medium(%): yeast extract 0.3, peptone 1.0, sodium chloride 0.5, pH 7.4–7.6, and sterilization for 20 min at 121 °C.

Solid broth medium(%): yeast extract 0.3, peptone 1.0, sodium chloride 0.5, agar 2.0, pH 7.0 ~ 7.2, and sterilization for 20 min at 121 °C.

Fermentation medium(%): SBM: bran(35) = 9:1, water 65, pH 5.4 ~ 6.6, and sterilization for 20 min at 121 °C.

144.2.3 Strain Breeding

144.2.3.1 Mutagenic Treatment

0.85 % LiCl and saline were added in the fresh slant HRBX0, and made the bacterial suspension of the concentration 10^8 cfu/ml with filtered by cotton after shocking [4]; HRBX0 was irradiated with the dosage of 100 Gy, 200 Gy, 300 Gy, 400 Gy, 500 Gy, and 600 Gy; The number of the bacterial suspension before and after radiation was counted by using the plate dilution method, the number of the unirradiated colony C and irradiated colony D was counted, and the death rate R and the positive mutation rate P was calculated

$$P = S/D \tag{1}$$

P positive mutation rate
S degree of hydrolysis after the irradiated to improve the number of colonies
D after the irradiated the sum total of colonies.

144.2.3.2 Screening Mutations

Plate after diluting the bacterial suspension of mutagenic treatment with appropriate method was spreaded, 30 °C for 2 days, the number of colonies was observed and counted. The single colony with big shape, smooth surface, surrounding sticky to screening was selected. The single colony of screening was

rescreened in the fermentation medium under the condition 37 °C, 60 h [5]. DH of SBM in the fermentation broth was determined and calculated, and the strains were saved which can make the DH of SBM higher.

144.2.3.3 Verify the Genetic Stability of Mutations

The rescreening strains were transferred continuously to culture to the fifth generation and ferment separately, DH of SBM was determined and calculated, the DH with their each original strain was compared, genetic stability was observed [6].

144.2.4 Culture Method

HRBX0 was grown in stock and maintained on Solid broth medium for 9 h at 37 °C. The cells were washed twice in sterile saline solution and inoculated to give a final inoculation of 10^7 cfu/ml. HRBX0 was grown in Liquid broth medium broth for 14 h at 37 °C, and then washed twice in sterile saline solution and inoculated to give a final inoculation of 10^7 cfu/ml.

144.2.5 Fermentation

Seven small-scale solid fermentations were performed in fermentation medium, including natural fermentation (carried out with the only microorganisms HRBX0), and six different induced fermentations with each of the microorganisms mentioned above in Sect. 144.2.3. Suspensions of fermentation medium were prepared and were allowed to ferment either spontaneously (no microorganisms added). The fermentation processes were carried out in a fermentor for 60 h at 37 °C. Samples were collected at times 0 and 60 h for pH and microbiological analyses. Fermented samples were freeze-dried for further analysis.

144.2.6 Analytical Methods

144.2.6.1 Determinations of Biomass

Method of absorbance values at 660 nm, method of dry weight.

144.2.6.2 Counts of Bacterial Suspension

The fermentation process was monitored by withdrawing samples at times 0 and 48 h using plate counts to determine changes in viable cells. It was measured by microscope count method and plate count method.

144.2.6.3 Analysis of Amino Acids by HPLC

Determination of amino acids was carried out by acid hydrolysis, derivatization, and HPLC quantification. In brief, 200 μl (0.2 mmol/ml) of DL-norleucine were added to 50 mg of sample as internal standard. Protein hydrolysis was carried out with 6 M HCl for 21 h at 110° C in a vacuum closed vial. Hydrolysates were dried under vacuum and rinsed twice with water. PITC (phenylisothiocyanate 99 %) was used for amino acid derivatization.

144.2.6.4 DH

Alpha-amino nitrogen (AN) was assayed in duplicate by the formol titration procedure. Total nitrogen (TN) was measured also in duplicate by the microK-jeldahl method. The percent degree of hydrolysis (DH) was calculated as in (1):

$$\% \text{ DH} = \text{An}_\text{h} - \text{AN}_\text{c}/\text{TN} \times \text{P}_\text{f} \times 100 \quad (2)$$

where An_h and AN_cwere the percent amino nitrogen of the hydrolysate and intact SBM, respectively. No significant difference was found between total nitrogen of the intact SBM and that of the hydrolysates.

Therefore, TN in Eq. (1) referred to the mean percent total nitrogen of the intact SBM solution and all hydrolysate samples, and P_f was a correction factor for side chain nitrogen which could not be converted to amino nitrogen by hydrolysis of peptide bonds. The P_f factor (0.777) was calculated from the amino acid profile of SBM.

144.3 Results and Discussion

144.3.1 Selection of the Dose of ^{60}Coγ

HRBX0 was irradiated with the dosage of 100 Gy, 200 Gy, 300 Gy, 400 Gy, 500 Gy, 600 Gy, and the death rate and the positive mutation rate were calculated, the results were shown in Figs. 144.1 and 144.2.

As shown in Fig. 144.1, different doses of rays all had the lethal effect to HRBX0, and death rate increased with the increasing of the radiation dose; when the radiation dose was 100 Gy, the death rate of the cells reached 68.25 %, and when 500 Gy, the death rate almost 100 %.

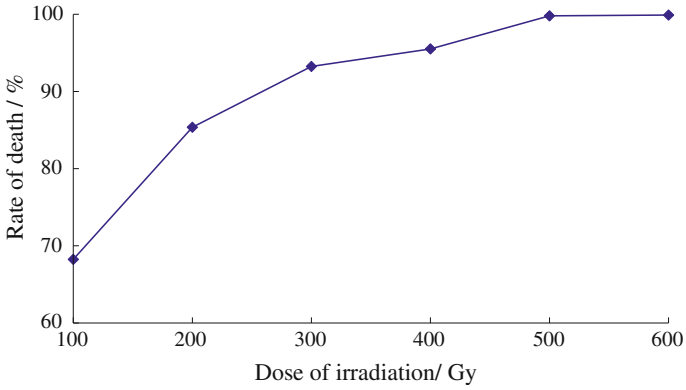

Fig. 144.1 Relationship between irradiation dose and rate of death

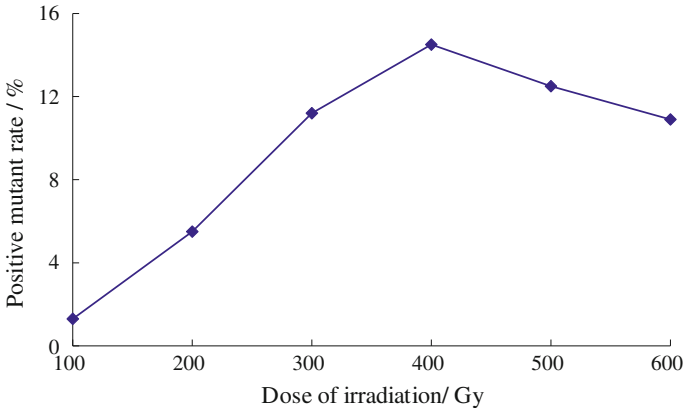

Fig. 144.2 Relationship between irradiation dose and positive mutation rate

As shown in Fig. 144.2, varying degrees of positive mutation rate of the strains appeared with different radiation doses, it showed that the mutagenesis by using ^{60}Coγ is effective; mutants had higher positive mutation rate when the radiation dose was 400 Gy or 500 Gy, especially when 400 Gy, positive mutation rate reached 14.5 %. In order to improve test efficiency, the mutants were selected which were irradiated with the radiation dose 400 Gy to fermentations test for screening the mutants of higher DH.

144.3.2 Result of ^{60}Coγ Irradiation

HRBX0 was irradiated with the dosage of 400 Gy, and the death rate and the positive mutation rate were calculated, the DH of SBM were obtained by fermentations test, the results were shown in Table 144.1.

Table 144.1 Result of $^{60}Co\gamma$ irradiation on HRBX0 strains

Irradiation dose/ Gy	Death rate/ %	Positive mutant rate/ %	DH (average value)/ %	Increase rate/ %
400	95.51	14.5	30.6	56.9

Table 144.2 Effect of generation on stability of degree of hydrolysis of mutation strains

Bacillus subtilis *natto*	Generation					
	1	2	3	4	5	Average value
HRBX0(DH)/%	19.3	19.0	19.7	19.3	19.5	19.36
HRBX6(DH)/%	31.1	30.5	30.2	30.8	30.7	30.62

Positive mutation rate of HRBX0 was 14.5 % with the optimized conditions of $^{60}Co\gamma$, the DH of SBM in solid-state fermentation of the mutant HRBX6 increased from original 19.5 to 30.6 %, increased by 56.9 %. The capacity of hydrolyzing soybean meal improved significantly higher than the original strain.

144.3.3 Result of Solid-State Fermentation and Stability Test with the Positive Mutant HRBX6

The positive mutant HRBX6 was obtained by screening, DH of defatted SBM with the fermentation experiments of the mutant after continuous cultivation for five generations were compared, the results were given in Table 144.2.

The positive mutation strain HRBX6 showed a good stably DH of defatted SBM with the experiments after continuous cultivation for five generations, the average of the DH increased from original 19.36 to 30.62 %, increased by 58.1 %. The capacity of the degree of hydrolysis was close from the fifth generation to first generation of mutant strain HRBX6, and it showed a good genetic stability of HRBX6.

144.4 Conclusion

Different doses of rays all have the lethal effect to HRBX0, and death rate increases with the increasing of the radiation dose; when the radiation dose is 100 Gy, the death rate of the cells reach 68.25 %, and when the radiation dose is 500 Gy, the death rate of the cells reach almost 100 %, and mutants have higher positive mutation rate when the radiation dose is 400 and 500 Gy, especially when 400 Gy, positive mutation rate reach 14.5 %.

HRBX0 was used as the original strain. It was processed by different doses of ^{60}Coγ, and then screened the mutant strain. The mutation strain HRBX6 with the high DH was obtained by different doses of ^{60}Coγ and screened the mutant strain, it showed a good genetic stability and the DH increased from original 19.36 to 30.62 %, increased by 58.1 %. ^{60}Coγ is an effective factor of mutagenic breeding of *Bacillus subtilis* natto.

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