Heavy-ion mutagenesis significantly enhances enduracidin production by *Streptomyces fungicidicus*

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To improve fermentative production of enduracidin, heavy-ion beams generated by the Heavy Ion Research Facility in Lanzhou (HIRFL), China, were employed for the first time to generate mutations in *Streptomyces fungicidicus*. Initial screening detected 44 positive mutants with larger inhibition zone, which were subsequently tested based on flask fermentation. Finally, 20 mutants showed 20% increase in enduracidin production, when compared with the original strain. Among them, enduracidin production by the three mutants (M13, M30, and M34) was significantly higher than that by the original strain. In particular, mutant M30 exhibited highest enduracidin production, which was 114% higher than that obtained with the original strain. Following culture optimization, the maximal enduracidin yield obtained by M30 reached 918.5 mg/L in 10 days, which was 34% higher than that noted in the control.

KEYWORDS

fermentation industry, heavy ion mutagenesis, mutation tool, screening process, *Streptomyces fungicidicus*

1 | INTRODUCTION

Enduracidin, produced by *Streptomyces fungicidicus*, is a major commercial antiparasitic agent widely used in agriculture, animal healthcare, and human healthcare owing to its high safety, low toxicity, low residue, unlikelihood to produce drug resistance, powerful bactericidal effects on most of the Gram-positive bacteria, and ability to promote animal growth [1,2]. In nature, *S. fungicidicus* can only accumulate low concentration of enduracidin; hence, further biotechnological advances are needed to enhance enduracidin production by *S. fungicidicus*. Currently, different strategies, including rational metabolic engineering and non-rational mutation screening strategies, have been employed to develop robust microbial strains with improved productivity [3]. Although the gene cluster responsible for the biosynthesis of enduracidin in *S. fungicidicus* has been studied [4], *S. fungicidicus* has not yet been subjected to typical rational metabolic engineering mainly owing to poor understanding

of enduracidin biosynthesis. Therefore, physical and chemical mutation methods are still preferred for obtaining promising *S. fungicidicus* strains.

Currently, several strategies have been applied to improve the enduracidin yield of *S. fungicidicus*, including mutation screening strategy [1] and pH shift feeding strategy [2]. It is noted that mutation screening strategy plays an important role in enduracidin fermentation industry. Heavy-ion beam irradiation is a newly developed mutagenesis method [5]. When compared with other traditional mutagenesis approaches, this method has several advantages such as higher mutation rate and wider mutational spectrum owing to its high linear energy transfer (LET; the energy transferred per unit length, KeV/ μ) [6]. To date, several robust microbial strains, including bacteria, fungi, and microalgae, have been developed using high-LET heavy-ion mutagenesis [5]. Although heavy-ion mutagenesis has been applied to Actinomycetes (such as *Streptomyces avermitilis*) [7], it has not yet been employed for obtaining robust *S. fungicidicus* with high enduracidin accumulation. In the present study, heavy-ion mutagenesis as a new microbial mutation tool was applied for the first time to generate

S. fungicidicus mutants. Based on multiple screening, *S. fungicidicus* mutants with the ability to accumulate high amount of enduracidin were generated. Furthermore, the productivity and genetic stability of the typical mutants were examined, and the culture conditions were also optimized.

2 | MATERIALS AND METHODS

2.1 | Strain and media

Original *S. fungicidicus* SG-01, which can produce enduracidin, was provided by biophysics lab of Institute of Modern Physics, Chinese Academy of Sciences. The strain was maintained on agar slant at 4°C. The agar slant medium contained (L⁻¹): maltose 8 g, yeast extract 0.8 g, tuna extract 0.8 g, tryptone 1 g, agar 20 g, and pH 6.8–7.0. *Bacillus subtilis* 63501 was purchased from China Institute of Veterinary Drug Control. The strain was maintained on beef extract peptone medium at 4°C and used as the indicator bacteria. The examination medium consisted of two layers with 10 mL medium, respectively. In brief, the over layer contained (L⁻¹): peptone 5 g, beef extract 5 g, NaCl 8 g, Na₂HPO₄ 2 g, agar 10 g and pH 7.0. The base layer contained (L⁻¹): peptone 5 g, beef extract 5 g, NaCl 8 g, Na₂HPO₄ 2 g, agar 20 g and pH 7.0. The seed medium contained (L⁻¹): glucose 30 g, corn steep liquor 30 g, yeast extract 5 g, Ca₂CO₃ 20 g, NaCl 8 g, and pH 7.5. The fermentation medium contained (L⁻¹): glucose 40 g, soluble starch 40 g, corn steep liquor 20 g, corn gluten meal 30 g, NaCl 15 g, NH₄Cl 5 g, Ca₂CO₃ 15 g and pH 6.9–7.1.

2.2 | Irradiation and screening process

The experimental protocol for irradiation and screening process of *S. fungicidicus* with carbon ion beams is shown in Fig. 1. In brief, original *S. fungicidicus* SG-01 was incubated on the agar slants at 28°C for 7 days, and *S. fungicidicus* SG-01 from slants was diluted with sterile saline water. Then, 1.0 mL suspension liquid was transferred into 35 mm irradiation dish. The colony-forming units/mL (CFU/mL) was maintained at 3×10^7 cells/mL. Bacteria suspension in irradiation dish was subjected to mutagenesis by 80 MeV/ μ carbon ions with the LET of 40 Kev/ μ in Heavy Ion Research Facility in Lanzhou (HIRFL). The irradiation doses were set as 0 Gy, 20 Gy, 40 Gy, 60 Gy, 80 Gy, and 100 Gy. Three parallel groups for each sample were irradiated at each dose.

After irradiation, the mutagen-treated and untreated aliquots of *S. fungicidicus* SG-01 suspensions were properly diluted and spread on agar plate medium, followed by cultivation at 28°C for 2 days. Then the single colony was punched respectively by a puncher with 6 mm of diameter and these agar blocks were cultured independently by transferring to

PRACTICAL APPLICATION

Heavy-ion mutagenesis significantly improved enduracidin production by *Streptomyces fungicidicus*. The robust *S. fungicidicus* mutant obtained in this study could be used as a potential host for improving enduracidin production via rational design. Besides, heavy-ion mutagenesis could be developed as a promising and convenient mutation tool for application in fermentation industry.

sterile empty culture dish at 28°C until 7 days. The agar blocks were put onto the examination medium with 0.2% *Bacillus subtilis* suspension (4×10^7 cells/mL) at 37°C for 18 h. Accordingly, through recording and comparing the diameter size of inhibition zone with control, the positive mutants were primarily selected based on bigger inhibition zone (above control 20%).

For rescreening, original *S. fungicidicus* SG-01 and its mutants with large inhibition zone were selected for shaking flask fermentation. The spore suspension (3×10^7 cells/mL) were transferred into 250 mL shake flasks with 50 mL seed medium on a SPH-311D rotary incubator (Shanghai Shiping Laboratory Equipment Co., Ltd, China) at 28°C and 220 rpm for 48 h. Then, 4 mL of the seed culture was transferred to 250 mL shake flasks with 50 mL fermentation medium, and fermented at 28°C for 8 days, with shaking at 220 rpm. The high-yield strains were selected and maintained on agar slants at 4°C for further experiments.

2.3 | The analysis of fermentation characteristics between original *Streptomyces fungicidicus* SG-01 and mutants

Both original *S. fungicidicus* SG-01 and its mutants were transferred into 250 mL shake flasks with 50 mL seed medium at 28°C and 220 rpm for 48 h. Then, 4 mL of the seed culture was transferred to 250 mL shake flasks with 50 mL fermentation medium, and fermented at 28°C and 220 rpm. The fermentation period was 10 days and fermentation characteristics including dry well weight (DCW), residual sugar content, and enduracidin production, were analyzed at the interval of 2 days.

2.4 | Media optimization

The effects of glucose, starch, corn steep liquor, KH₂PO₄, and arginine on enduracidin production were tested to determine the optimum concentration for enduracidin production of the mutant M30. The test range of glucose concentration was designed from 2 to 10% (w/v), starch and corn steep liquor concentration were designed to range from 1 to 7% and 1–5%,

FIGURE 1 The experimental protocol for irradiation and screening process of *S. fungicidicus* with carbon ion beams

as well as KH_2PO_4 and the arginine concentration were also designed to range from 0–0.05% and 0–0.2% respectively.

2.5 | Analytical methods

The biomass was estimated by DCW that 5 mL samples were centrifuged (4,000 r, 10 min) on 3–5N centrifuge (Hunan Hengnuo instrument equipment Co., Ltd., China), washed with distilled water in triplicate, and dried at 60°C until constant weight. Residual sugar content was determined with 3, 5-dinitrosalicylic acid method (DNS method) [8]. The production of enduracidin was determined by the Waters e2695 HPLC (Waters, America). The freshly mycelia was harvested by centrifuging 5 mL samples (4,000 r, 10 min), and resuspended in 5 mL acetone extract (acetone: 2 M HCl: H_2O = 20:1:20). The mixture was sonicated with an ultrasonic wave for 30 min at 360 W on VGT-2013QTD ultrasonic cleaner (Shenzhen VGT Co., Ltd., China), and then centrifuged at 4000 r for 10 min. The supernatant was collected and filtered through a 0.22 μm syringe filter prior to determine the production of enduracidin. HPLC was implemented by using a C_{18} reverse column (4.6 $\times$ 150 mm, 5 μm ; Lanzhou Zhongke Kaidi Chemical New-tech Co., Ltd., China), and the mobile phase was in 30% acetonitrile and 70% 5M NaH_2PO_4 with pH adjusted to 4.5 by phosphoric acid, at a flow rate of 1.0 mL/min. The detection wavelength was at 267 nm. Before analysis, sample volume and column temperature were set as 20 μL and 30°C, respectively. All experiments were conducted in triplicates.

3 | RESULTS

3.1 | Lethality of *S. fungicidicus* spores irradiated with carbon ion beams

Owing to the lack of referenced conditions for heavy-ion mutagenesis of *S. fungicidicus*, the effects of five heavy-ion irradiation doses (20, 40, 60, 80, and 100 Gy, respectively)

FIGURE 2 Effects of exposure dose on lethality rate of *S. fungicidicus* SG-01. The lethality rate can be calculated using the number of clones in control plates without heavy-ion beam treatment as 100%. The results were performed in three parallel replicates. The error bars in the figure indicate the standard deviations of three parallel replicates

with LET of 40 KeV/μ on cell lethality were examined. The lethality of *S. fungicidicus* exposed to various doses of high-LET heavy carbon ion mutagenesis was shown in Fig. 2. The relationship between the lethality of *S. fungicidicus* and carbon ion irradiation dose fitted a linear regression equation ($Y = 1.0167X - 6.5743$, $R^2 = 0.96$), indicating that the death rate of cells increased with increasing radiation dosage from 20 to 100 Gy. The maximum lethal rate reached 93.24% at a radiation dosage of 100 Gy. The mutants obtained following 20, 40, 60, 80, and 100 Gy irradiation were screened.

3.2 | Selection of mutants with high enduracidin production

For the initial screening of efficient mutants, agar block screening process was employed. In brief, the colonies on solid medium were incubated at 28°C for 2 days and then punched using a puncher with 6-mm diameter. Then, these

FIGURE 3 (A) Initial screening process based on inhibition zone diameter. (B) The second round screening based on flask fermentation. (C) Obtaining robust *S. fungicidicus* mutants. (D) The genetic stability of three mutants. The error bars in the figure indicate the standard deviations of three parallel replicates, and $p < 0.05$ indicated a significant difference and $p < 0.01$ indicated a highly significant difference

punched agar lumps with the colonies were placed into sterile empty culture plates with 12-mm diameter and incubated at 28°C for 7 days. Subsequently, these punched agar lumps with colonies were transferred onto solid medium containing *B. subtilis* at 37°C. After cultivation for 18 h, obvious inhibition zone was observed and the zone diameter was measured and compared with that noted in the control. Following carbon ion irradiation, about 900 bacterial colonies were selected and subjected to the initial screening process (Fig. 3A). A total of 44 positive mutants with larger inhibition zone were screened and then examined based on flask fermentation. The results showed that 20 mutants presented 20% increase in enduracidin production, when compared with the original strain (Fig. 3B). Subsequently, these 20 mutants were screened based on flask fermentation. Following multiple rounds of screening, three mutants with significantly high enduracidin production (M13, M30, and M34), when compared with that noted in the original strain, were detected. In particular, mutant M30 exhibited the highest capacity for producing enduracidin, which was 114% higher than that produced by the original strain (Fig. 3C).

Subsequently, the genetic stability of these three mutants was investigated. The three mutants showed

stable enduracidin production through five consecutive generations in shake flask (8 days) (Fig. 3D). Furthermore, HPLC analysis revealed the accumulation of enduracidin A and B in mutant M30 after 8 days of cultivation in fermentation medium (Fig. 4). Besides, mutant M30 exhibited faster spore production after 7 days on solid medium at 28°C, when compared with the original strain (Fig. 5). These results indicated that heavy-ion mutagenesis is suitable for improving enduracidin production by *S. fungicidicus*.

3.3 | Comprehensive fermentation characteristics between original strain and mutants

The three mutants (M13, M30, and M34) were inoculated into 250-mL flasks containing 50 mL of fermentation medium to determine their comprehensive fermentation characteristics. The original strain was employed as the control, and the experiments were conducted in triplicates. In particular, several fermentation indices were included, namely, enduracidin concentration, reducing sugar concentration, and DCW. As shown in Fig. 6A, all the mutants

FIGURE 4 HPLC chromatogram of enduracidin, (A) reference substance for enduracidin, (B) enduracidin from fermentation product

FIGURE 5 Typical images of the spore morphology between original strain and mutants

exhibited significant increase in enduracidin accumulation at different fermentation periods, when compared with the original strain. The maximal enduracidin concentration produced by mutant M30 reached 685.9 mg/L on day 10.

The DCW of mutants M13 and M34 increased during the entire fermentation period, when compared with that

of the original strain. However, mutant M30 showed lower DCW in the initial fermentation phase (0–6 days), whereas mutant M30 presented rapid increase in DCW in the later fermentation phase (6–10 days) (Fig. 6B). As shown in Fig. 6C, the highest specific enduracidin production rate exhibited by these three mutants was significantly elevated, when compared with that presented by the original strain;

FIGURE 6 Comprehensive fermentation characteristics between original strain and mutants. (A) Enduracidin concentration. (B) Time profiles of cell growth. (C) Specific enduracidin production rate. (D) Reducing sugar concentration. The error bars in the figure indicate the standard deviations of three parallel replicates

in particular, mutant M30 showed the maximum specific enduracidin production rate (4.36 mg/(L d)), which was 2.48-fold higher than that achieved with the original strain.

The results of reducing sugar consumption by the mutants and original strain are shown in Fig. 6D. Both mutants M13 and M34 showed no significant difference in the reducing sugar consumption rate, when compared with the original strain during the entire fermentation period. However, mutant M13 presented slower glucose consumption rate than the original strain.

3.4 | Media optimization

Fermentation media could affect the production of primary and secondary metabolites, and the supply of essential nutrients is necessary for the growth of microbial cells or product formation in industrial-scale fermentation [9]. In this study, to further improve enduracidin production by mutant M30, the effects of medium components, including concentrations of

added glucose, starch, corn steep liquor, KH_2PO_4 , and arginine, on enduracidin production were evaluated. As shown in Fig. 7A–C, the optimal concentrations of glucose, starch, and corn steep liquor were 4, 4, and 2%, respectively, which were the same as those of the three components in the initial medium. In addition, enduracidin production by mutant M30 was significantly enhanced with the addition of KH_2PO_4 and arginine (Fig. 7D–E), and the optimal concentrations of KH_2PO_4 and arginine were 0.02 and 0.1%, respectively. Similar results have also been reported by Hatano et al. [10] and Lounes et al. [11]. Under the optimal medium conditions, the maximal enduracidin production by mutant M30 reached 918.5 mg/L on day 10, which was 34% higher than that noted in the initial medium (Fig. 7F).

In a previous study, *Streptomyces* sp. MC079 isolated from deep-sea sediment [2] was reported to produce 61.37 mg/L enduracidin with 16.176 mg/L d productivity via a pH shift feeding strategy. Furthermore, Zhang et al. [1] obtained mutant *S. fungicidicus* S-224 by ARTP mutagenesis and

FIGURE 7 Media optimization of M30 strain

achieved 1.58 g/L enduracidin yield, while Mou et al. [12] constructed an excellent genetically modified strain of *S. fungicidicus* L-M2 (Arg43) that could produce 1456 U/mL enduracidin. However, it must be noted that the original strains used in the later two studies showed high enduracidin-producing ability. In the present study, the original strain could only accumulate 339.7 mg/L enduracidin in 10 days, whereas the promising mutant M30 produced 918.5 mg/L enduracidin in 10 days following medium optimization. In future, multiple heavy-ion mutagenesis workflows should be

conducted to further enhance the enduracidin-producing ability of mutant M30.

4 | DISCUSSION

Currently, traditional genetic engineering tools and large biosynthetic gene clusters have been major obstacles to metabolic engineering of *S. fungicidicus* for enduracidin biosynthesis. Therefore, it is particularly necessary to find

alternative approaches to develop robust *S. fungicidicus* strains with high enduracidin production capacity. Heavy-ion mutagenesis is an effective methodology for generating mutations in plants and microorganisms, with high mutation rate and broad spectrum because of its distinct physical and biological advantages [5,13]. Heavy-ion mutagenesis can cause direct DNA damage in the irradiated microbial strains through thermal, dynamic, and ionization effects, as well as indirect DNA damage by reactive oxygen species produced by the accelerated particles of heavy-ion beams along their track region. These DNA damage mechanisms induced by heavy-ion beams are beneficial for generating microbial mutants, with higher possibility of obtaining robust microbial mutants. Hence, in the present study, heavy-ion irradiation mutagenesis was employed to develop robust *S. fungicidicus* mutants with enhanced enduracidin accumulation capacity. Moreover, to the best of our knowledge, this study is the first to apply heavy-ion mutagenesis on *S. fungicidicus*.

The results obtained revealed the following: (1) Heavy-ion mutagenesis had obvious mutation effects on *S. fungicidicus*, with the lethality of irradiation enhancing with increasing irradiation dose, indicating that *S. fungicidicus* was sensitive to high LET carbon ion beams. (2) Based on multiple rounds of screening, three mutants (M13, M30, and M34) that showed obviously higher enduracidin accumulation than the original strain were selected. However, the screening process for robust *S. fungicidicus* strain, consisting of agar block preliminary screening, flask fermentation rescreening, and HPLC identification for enduracidin accumulation, was time-consuming because of the large number of strains that had to be tested. Hence, it is necessary to establish relatively simple screening methods to enhance screening throughput and efficiently obtain positive strains. Furthermore, the colony development (such as spore production rate) of robust *S. fungicidicus* mutants was different from that of the original strain. In previous studies, Actinomycetes colony development has been noted to influence its antibiotic production [12,14]. These findings further suggest that colony development changes in *S. fungicidicus* could be a target phenotype for rapid screening of robust *S. fungicidicus* mutants via heavy-ion mutagenesis. (3) The LET used in the present study was 40 Kev/ μ , which produced several promising mutants. In previous studies, heavy-ion beams with different LET have been found to exert varied biological effects on plants [15] and microbial strains [16]. Hence, the effects of heavy-ion beams with different LETs on *S. fungicidicus* must be further examined, because the LET value might affect double-strand breaks induction efficiency in irradiated cells [17]. It has been indicated that the biosynthesis of enduracidin in *S. fungicidicus* is mainly controlled by gene cluster, including endA, endB, endC, and endD [4]. Based on these findings and the complexity of enduracidin biosynthesis in *S. fungicidicus*, it can be suggested that heavy-ion mutagenesis probably induced

multiple gene mutation sites in *S. fungicidicus*, or totally changed the metabolic network involving enduracidin accumulation in *S. fungicidicus* mutants. Therefore, further detailed investigations on the changes in the metabolic pathways and precise mutation gene sites of *S. fungicidicus* mutants should be conducted using molecular biotechnology tools such as genomics, proteomics, and metabolomics.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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