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RESEARCH PAPER

Mutation Breeding of High Avermectin B_{1a}-producing Strain by the Combination of High Energy Carbon Heavy Ion Irradiation and Sodium Nitrite Mutagenesis Based on High Throughput Screening

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Abstract Microbial mutation breeding has been widely used because it is one of the most efficient and practical breeding strategies in the fermentation industry. However, different mutagenesis methods cause various degrees of DNA damage to individual microorganisms, which lead to diverse characteristics of the mutants. In this study, the effects of four different mutagenesis methods on the mutation breeding of *Streptomyces avermitilis* for improving avermectin B_{1a} production were investigated with an optimized liquid microtiter plate (MTP) culture system. First, an effective and feasible MTP system for mutant strain screening was evaluated through the optimization of the oxygen transfer rate and rapid titer determination. Then, high energy carbon heavy ion irradiation, diethyl sulfate, ultraviolet- (UV) irradiation combined with lithium chloride, and sodium nitrite were used as the mutagens for mutation breeding, respectively. Results showed that carbon heavy ion irradiation had the advantages of possessing the highest positive mutation rate and mean-production of positive mutant strains in the first generation. Sodium nitrite treatment resulted in mutant strains with better inherited stability than the other three methods. Through the combined treatment of carbon heavy ion irradiation and sodium nitrite treatment, an inherit-stable mutant *S. avermitilis* S-233 with high avermectin B_{1a} production was successfully obtained. The fermentation verification in a 500-liter (L) bioreactor demonstrated that the avermectin B_{1a} produced by mutant *S. avermitilis* S-233 reached 6818 μg/mL, which was 23.8% higher than that of parent strains.

Keywords: avermectin B_{1a}, miniaturization culture, evaluation of mutagenesis method, high-throughput screening

1. Introduction

As a kind of agro-antibiotics produced by *Streptomyces avermitilis*, avermectins are widely used as insecticidal agents [1]. They are a sequence of 16-member pentacyclic compounds with a disaccharide of methylated deoxysugar L-oleandrose polyketides [2,3]. Among the eight congeners of avermectins, C5, C22, C23 and C26 portray different structures, and only avermectin B_{1a} is widely used due to its powerful insecticidal and anthelminthic capabilities [4,5]. Despite the accessibility of the entire genome sequence of *S. avermitilis*, including the gene cluster for the biosynthesis of avermectin [6,7], and advances in the fundamental studies on the biosynthetic pathway of avermectins and its regulatory mechanism [8,9], the functional analysis of genes, especially those involved in avermectin production, is still in progress [10]. Manipulation of one or two genes does not readily lead to a mutant with a high avermectin B_{1a} yield for industrial application [11].

Nowadays, the biopharmaceutical industry has a great deal of interest in high-throughput and reduced cell culture platforms that facilitate the screening of mutant strains and drugs with significant time and labor savings [12,13].

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Moreover, microtiter plates (MTP) as miniature cell culture devices are popular in high-throughput experiments for optimization and screening [14,15]. High-throughput screening combined with random mutagenesis is a powerful way to search for desirable phenotypes (*e.g.*, high productivity, high growth rates, and robustness).

Mutagenesis strategies have been studied and applied thus far by using physical (*e.g.*, γ-rays, X-rays, UV light, particle radiation, *etc.*) or chemical (*e.g.*, alkylating agents, azides, *etc.*) methods [16]. Such random mutagenesis strategies are reported to be one of the most efficient, practical, and useful breeding strategies in the fermentation industry [17]. Mutants with various characteristics are generated by a complex process. Generally, DNA damage is recognized as the first step in the induction of a mutation. The SOS regulatory network then plays a key role in DNA damage repair and lesion DNA synthesis [18,19]. These mechanisms enables cell survival after DNA damage by strictly repairing or creating an expedient process (*i.e.*, a random dNTP placed opposite to the damaged site to ensure continuous replication) for inducing mutation [20].

In this work, a liquid MTP culture method was optimized for *Streptomyces avermitilis* based on the oxygen transfer rate and rapid titer determination. Then a developed high-throughput culture system was used to evaluate four different mutagenesis methods: high energy carbon heavy ion irradiation (HECHI), diethyl sulfate (DES), UV-irradiation combined with lithium chloride, and sodium nitrite mutagenesis. About 1,000 mutants per investigated mutagenesis method were studied to conduct a comparative evaluation and statistical analysis for mutagenesis quality assessment. Next, HECHI combined with sodium nitrite was conducted as an appropriate way to increase the avermectin B_{1a} production in the consideration of both the positive mutation rate and inherited stability.

2. Materials and Methods

2.1. Strain

S.avermitilis-102, an industrial strain supplied by Qilu pharmacy (Inner Mongolia) Co. Ltd., was grown on a YMG agar medium (per liter: 4 g of starch soluble, 3 g of yeast extract, 10 g of malt extract, 5 mg of $CoCl_2$, 20 g of agar; pH7.3).

2.2. Medium

The seed medium contained (per liter) 30 grams (g) of corn starch, 8 g of soya flour, 10 g of peanut meal, 4 g of yeast extract, 0.03 g of $CoCl_2$, and 0.04 g of α-amylase. The pH of the seed medium was adjusted to 7.2 with 3 mol/L NaOH before autoclaving. The fermentation medium for the shake flask and fermenter contained (per liter) 120 g of corn starch, 28 g of soya flour, 10 g of yeast powder, 0.25 g of $(NH_4)_2SO_4$, 0.8 g of $CaCO_3$, 0.02 g of $CoCl_2$, 0.022 g of $NaMoO_4$, 0.0022 g of $MnSO_4$, and 0.1 g of α-amylase. The medium pH was adjusted to 7.5 with 3 mol/L NaOH before autoclaving. The fermentation medium used for MTPs was obtained by a 2-fold diluting of the fermentation medium for each shake flask with deionized water. The medium pH was also adjusted to 7.5 and 0.1 g of α-amylase was used to gelatinize corn starch before autoclaving.

2.3. Cultivation conditions

2.3.1. Traditional culture in shake flask

S. avermitilis was pre-cultured in a 500 mL shake flask containing 60 mL of seed medium at 28°C and 230 rpm for 40 h. The 500 mL shake flasks containing 40 mL of fermentation medium with 5% (v/v) inoculation quantity were cultivated at 28°C on a rotary shaker at 230 rpm for 240 h. The samples were taken every 24 h for analyses of the various process parameters.

2.3.2. Micro-culture in 48-deep MTPs

S. avermitilis spores were pre-cultured with a sterile toothpick into 48-deep MTPs containing 1 mL of seed medium per well for 38 h. A 5% (v/v) inoculation quantity was inoculated in the fermentation medium with 1.0 mL per well. The fermentation MTPs were cultivated at 28°C on a rotary shaker at 230 rpm for 240 h.

2.3.3. 500 L bioreactor studies

The production of avermectin was carried out in a 500 L fermenter with 300 L of fermentation medium for 312 h. Before sterilization, the pH was adjusted to 6.8 by 3 mol/L NaOH. The 30 L of seed culture was prepared in a 50 L fermenter. The conventional turbine blade impeller with six blades was implanted on all fermenters in this study. The mycelium from the agar slant was inoculated into the 50 L fermenter for 38 ~ 48 h. The packed mycelial volume (PMV, %v/v) was used to represent the biomass. After the PMV reached 30%, it was transferred into the next fermenter. Then 15 L of the seed culture from the 50 L seed fermenter was inoculated into a 500 L fermenter sterilely, and the initial impeller speed was adjusted to 300 rpm at a temperature of 28°C. The pH was maintained within a certain range with a 3 mol/L HCl or NaOH solution. Samples were monitored for every 12 h until the end of fermentation.

2.4. Analytical methods

2.4.1. Volumetric oxygen transfer coefficient (K_La) analysis

The data quality and reproducibility of aerobic microorganism

cultures in MTPs are greatly affected by mixing and aeration [21]. K_La (/h) is a proper index indicating the effect of aeration on MTPs, which can be determined by the optical sulfite oxidation method [22] in 48 MTPs and shake flasks. The filling volume was 600, 800, 1,000, 1,200, and 1,400 μL per well of each 48-deep MTP. Each 500-mL shake flask contained 40 mL of sulfite solution system. All of the experiments were performed at 230 rpm and the data represented the mean values of the three MTPs and six shake flasks.

2.4.2. Biomass concentration measurements

The PMV was obtained directly from the volume fraction of the packed mycelia by centrifuging 10 mL of the broth in conic scaled tubes at 4,000 rpm for 20 min.

To conduct cell dry weight analysis, 10 mL of fermentation broth was centrifuged at 4,000 rpm for 20 min. Then, the cell pellet was washed twice with deionized water and dried at 105ºC until it achieved a constant weight.

2.4.3. Sugar measurements

Total sugar and reducing sugar were both determined with a 3- and 5-nitro salicylic acid reagent colorimetry [23].

2.4.4. Assay of avermectin B_{1a} concentration

The production of avermectin B_{1a} was determined by a high-performance liquid chromatography (HPLC) (Agilent 1100 Series, USA). The samples were separated on an Agilent C_{18} column and eluted with methanol/water (88:12, v/v) at a flow rate of 1 mL/min, at 30°C and an automatic 20 μL injector with a UV detector at 246 nm. A 9-fold volume of methanol was added to the sample. The mixture was shaken for 30 min to extract avermectin B_{1a} from the mycelia. A 20 μL sample of the methanol extract was tested.

2.4.5. High-throughput assay for avermectin B_{1a}

A standard curve was generated using 150 μL of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 240, and 260 mg/L of commercially available avermectins (96% B_{1a} and 4%B_{1b}) based on the avermectin titer and OD_{246} value. Each well was added with 2 mL of methanol for the extraction of avermectins. After extracting for 4 h, supernatants were obtained by centrifuging at 4,000 rpm for 20 min. Samples were diluted with methanol into the linear range of detection.

2.5. Mutagenesis conditions

2.5.1. DES mutagenesis

The determination of the DES treatment concentration was as follows. The spore suspension was made by washing the fresh slant with 10 mL of sterile water (containing approximately 1.2×10^7 spores). The spore suspension (4 mL) was added to a certain volume of 20% (v/v) DES ethanol solution to create a final concentration of 0.25, 0.375, and 0.5% that was gently homogenized with glass beads in a 25 mL shake flask that was shook for about 30 min. After each treatment, 150 μL of 25% (w/v) sodium thiosulfate was added to the reaction mixture to stop the mutagenic process.

2.5.2. Sodium nitrite mutagenesis

The spore suspension (4 mL) was added to 2 mL of the sodium nitrite solution to create a final concentration of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mol/L. The mixture was gently homogenized with glass beads in a 50 mL shake flask that was shook for about 10 min.

2.5.3. UV-irradiation and lithium chloride mutagenesis

The determination of the UV-irradiation time was calculated in the following manner. The spore suspension (4 mL) was collected and then exposed to 15 W of UV irradiation at 254 nm for 1, 2, 3 or 4 minutes at a distance of 20 cm in a petri dish. Then, an isometric 1.2% lithium chloride solution was added to the petri dish, contacted and mixed for 20 min.

2.5.4. HECHI irradiation

HECHI irradiation is a type of high linear energy transfer (LET) irradiation that is widely used as a mutation method used with plant cells in order to improve products [24,25]. The determination of the HECHI irradiation dose was developed in the following manner. A volume of 2 mL of the spore suspension was pipetted into a 30 mm petri dish and irradiated by HECHI. The carbon ion energy was 80.55 MeV/u, and the dose rate was 4 Gy/min. The irradiating doses of the HECHI were 20, 40, 50, 60, and 80 Gy.

2.5.5. HECHI irradiation combined with sodium nitrite mutagenesis

The spore suspension (2 mL) was collected and then exposed to HECHI irradiation at a 60-Gy radiation dose. Next, 1 mL of the 1.2 mol/L sodium nitrite solution was added to the spore suspension after HECHI irradiation, which resulted in a final concentration at 0.4 mol/L. The mixture was gently homogenized with glass beads in a 50 mL shake flask that was shook for about 10 min.

Positive Mutant Rate

$$Positive\ mutant\ rate\ (\%) = \frac{positive\ mutants}{total\ selected\ colonies} \times 100\%$$

Positive mutants were defined as those with a higher avermectin B_{1a} production than the parent strain.

3. Results and Discussion

3.1. Optimization of the liquid microtiter plate (MTP) culture system for the high-throughput screening mutants of *S. avermitilis*

3.1.1. Oxygen transfer selection

The K_La in the 48-well microtiter plates were studied at different working volumes (*i.e.,* 600, 800, 1,000, 1,200, and 1,400 μL) at 230 rpm on a rotary shaker, which was calculated to be 141.6, 124.6, 91.8, 66.2 and 58.3/h, respectively. The inter-well reproducibility was fairly good with a 5% relative standard deviation (RSD). The K_La of the shake flasks (40 mL working volume/500 mL total volume) were 88.74/h, which suggested that the best filling volumes can be chosen from the range of 600 ~ 1,000 μL. Considering the high evaporation rate (approximately 32.5%) during the 10 day culture process, 1,000 μL was chosen as the working volume of the 48-deep MTPs.

During the culture of *S. avermitilis* in 48-deep MTPs, a slight disturbance outside would have a great impact on the cultivating system due to the low liquid medium volume, large surface tension, and gas-liquid contact area [21,26]. Therefore, the parallelism of each well was difficult to control especially for *S. avermitilis* that formed mycelium pellets easily. As such, soya flour and a combination of yeast powder and corn starch were used in this study as the nitrogen and carbon sources, respectively. Since soya flour and yeast powder have a large dimension and corn starch increases the viscosity of the fermentation broth, the oxygen dissolution was negatively affected. Therefore, the fermentation medium of the MTPs was optimized in order to improve the oxygen transfer rate and parallelism of each well. The first protocol, soya flour and yeast powder were replaced by inorganic nitrogen and ammonium sulfate based upon the content of the nitrogen (N) atom. The second, third and fourth fermentation medium were diluted 1.5-, 2- and 2.5-fold, respectively, with deionized water. Fifth, the concentration of soya flour and yeast powder was halved in the fermentation. As shown in Table 1, the values of the RSD of the 2 and 2.5-fold diluted medium were below 5%, which suggested that the parallelism between the wells was getting increasingly better with the dilution of the fermentation medium. Unexpectedly, the production of the diluted medium was higher than that of the original medium, which was likely due to the low viscosity (Table 1) contributed to the increased dissolved oxygen in the fermentation process. The low titer of the first protocol might be due to the lack of some microelements in the inorganic nitrogen; while the low titer of the fifth protocol was likely due to the relatively larger viscosity caused by the corn starch relative to the third protocol.

As displayed in Table 1, the growth characteristics of a strain in a 2-fold dilution medium were similar to that in the shake flasks, which suggested that the 48-deep MTPs with a 2-fold dilution medium could replace the shake flasks as a curtailed tool to culture *S. avermitilis*.

3.1.2. Rapid titer determination assay of avermectin B_{1a} in broth

The HPLC assay is not suitable for a high-throughput quantitative analysis because of its high cost and low efficiency. A simple and rapid high throughput assay should be constructed to determine large numbers of avermectin samples simultaneously. We found the peak area of avermectin B_{1a} accounted for about 32% of the whole peak area in the HPLC detection of the avermectin fermentation broth extract in 240 h. Accordingly, the avermectin B_{1a} concentration may be indirectly detected by the absorbance of the avermectin liquid at 246 nm with the help of the microplate reader. A standard curve with $R^2 = 0.998$ was generated when using commercially available avermectins as the standard substance. The avermectin B_{1a} concentration of the fermentation broth can be formulated as:

$$Y^* = (OD_{246} + 0.0541)/0.0116 \times 0.32.$$

*avermectin B_{1a} concentration

To examine whether the UV assay could represent avermectin B_{1a} production, the correlation between the UV and HPLC assays was further investigated. The production of forty randomly selected strains was determined by both the UV and HPLC assays. As shown in Fig. 1, the UV

Table 1. Comparison of multiple parameters in different culture condition

	MTP						FLASK
Fermentation medium	Original medium	Inorganic nitrogen	1.5-fold dilution	2-fold dilution	2.5-fold dilution	On-half [b]	Original medium
Viscosity (pa*s)[a]	550	187	296	123	77	408	387
Production (μg/mL)	1,210	90	2,260	2,330	1,980	1,300	5,170
RSD (%) of production	11.8	7.5	6.1	4.3	4.0	12.9	/
Specific production (mg/gDW)	14.4	9.1	5.1	42.1	33.1	24.7	46.4

[a]Viscosity was measured at 48h in the fermentation process, when viscosity had the largest value.
[b]With the half organic nitrogen source concentration compared with control.

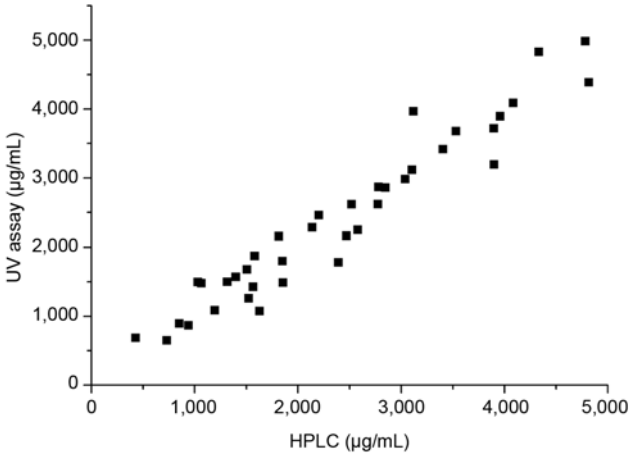


Fig. 1. Avermectin B_{1a} production determined by UV assay and HPLC.

results were significantly correlated with those assayed by HPLC. The Pearson coefficient between the UV assay and HPLC was 0.96. Moreover, the recovery experiments of the UV assay were calculated by adding a known avermectin B_{1a} concentration (1,000 μg/mL) broth to 3 samples (Table 2). Obviously, the high-throughput UV assay can be effectively used for the determination of the avermectin titer in the high-throughput system.

3.2. Evaluation of different mutagenesis methods used for mutation breeding of *S. avermitilis*

Different mutagenesis causes various degrees of DNA damage to microorganisms that can lead to diverse characteristics of the mutants [18]. We applied mutagenesis such as HECHI irradiation, DES, sodium nitrite, and UV-irradiation combined with the lithium chloride method to *S. avermitilis*. The DES mutagenesis induced a dose-dependent lethality rate in *S. avermitilis* with respect to the treatment concentration. After treatment with the DES concentrations of 0.25, 0.37, and 0.50%, the lethality rate of the treated spores increased to 61.5, 82.6, and 93.6%, respectively. The highest positive mutation rate was obtained when the mortality rate of the microorganism was as high as 80% according to the report [27]. Therefore, the DES concentration was calculated as 0.37%. In the case of other mutagenesis methods, the lethality rate was 83.2% with the sodium nitrite concentration of 0.4 mol/L. And it came to 80.6% when the spores were exposed to the ultraviolet for 1 min and contacted with 1.2% lithium chloride solution for 20 min. The HECHI irradiation dose was 60 Gy with a lethality rate of 81.2%.

For each method, about 1,000 ripe bacterial colonies were generated with sterile toothpicks from plates and transferred to 48-deep MTPs for cultivation. As shown in Fig. 2, the interval numbers of the production distribution were examined after the MTPs preliminary screening and the positive mutant rate varied with mutagenesis. The maximal positive mutation rate of 28.1% was obtained by HECHI irradiation (Fig. 2F). Among the 981 selected strains, 74 strains increased by at least 20% (Fig. 2A). However, the positive mutation rate of DES was 17.5%, and the number of mutant strains with production improvement of more than 20% was 54 out of a total of 972 studied strains (Fig. 2B). Regarding the sodium nitrite mutagenesis, 65 mutant strains attained production above 120% among 969 strains with the second max positive mutation rate as 21.0% (Figs. 2C and 2F). The minimal positive mutation rate was obtained by UV-irradiation combined with 12.7% lithium chloride; only 29 mutants increased by 20% or more among the 960 strains (Fig. 2D).

The mutants with production that increased by more than 20% were selected after the preliminary screening and their propagating stability were performed using a shake flask test. The number of mutants from the HECHI irradiation, DES, sodium nitrite and UV-irradiation combined with lithium chloride was 74, 54, 65 and 29, respectively. Fig. 3 provided the relative production and number of positive mutants of each mutagenesis. However, both the production and number of positive mutant strains of each mutagenesis decreased as the serial passages increased. In the first generation, the mean production of the positive mutants was 137.1, 123.9, 120.7, and 118.8% after HECHI irradiation, DES, sodium nitrite and UV-irradiation combined with lithium chloride mutagenesis, respectively. The number of mutants with production that increased by at least 20% was 42, 23, 23, and 9, respectively (Fig. 3F). However, the mean production of the positive mutants decreased to 116.8, 116.0, 119.4 and 113.1%, respectively, and only 6, 7, 14 and 2 mutants, respectively, attained production exceeded 20% in the third generation. As for the fifth generation, there were no mutants that increased by more than 20% and the mean production of the positive mutants was 103.8, 106.1, 108.1, and 105.5%, respectively. Although the positive mutant

Table 2. The recovery experiments of UV assay

Sample	B_{1a}concentration (μg/mL)	Determined concentration (μg/mL)	Reduced (μg/mL)	Recovery (%)
1	1,500	2,490 ± 40	990	99
2	2,500	3,540 ± 50	1,040	104
3	3,500	4,530 ± 60	1,030	103

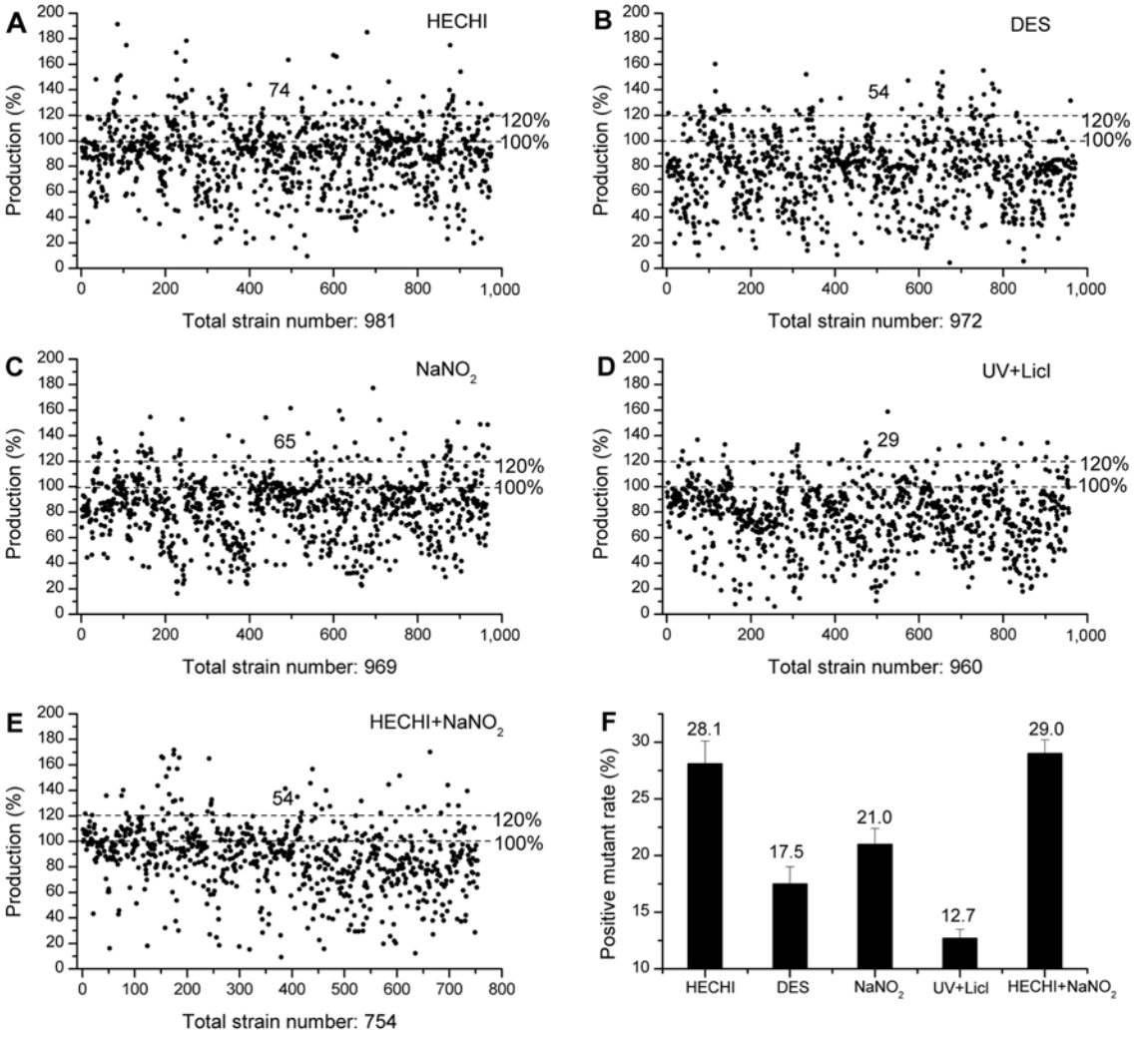


Fig. 2. Avermectin B_{1a} production point distribution and positive mutant rate of different mutagenesis method. (A) Carbon heavy ion irradiation, (B) DES, (C) sodium nitrite, (D) UV-irradiation combined with lithium chloride, (E) HECHI irradiation combined with sodium nitrite, (F) positive mutant rate of different mutagenesis method.

strains from HECHI irradiation had the highest mean production and the most positive mutant number in the first generation, the stability was not good in the third and fifth generation. There were only 12 strains with an improved production and the highest production was 108.5% when passed to the fifth generation (Fig. 3A). DES and UV-irradiation combined with lithium chloride mutant strains had a problem similar to HECHI irradiation (Figs. 3B and 3D). In the case of sodium nitrite mutagenesis, the production of mutants in the fifth generation was relatively higher than other ways, and the highest production was 114.8%. Notably, there were still 29 positive mutant strains among the third generation; 14 of those strains increased by over 20%, which was at least twice as much as that produced by the other approaches (Fig. 3C).

Hence, with the consideration of both the positive mutation rate and inherited stability, *S. avermitilis* was mutated using HECHI irradiation combined with sodium nitrite to obtain mutant strains with higher avermectin B_{1a} production. A total of 754 colonies were chosen from plates and transferred to 48-deep MTPs for cultivation after mutagenesis. The interval numbers of production and positive mutant rates were attained after the preliminary screening (Fig. 2E). The positive mutation rate of the new mutagenesis was as high as 29.0% (Fig. 2F), and the number of mutant strains with the production improved by more than 20% was 54. Next, the stability of the high-yielding mutants was also tested (Fig. 3E). The mean production of the positive mutants was 122.6, 118.8, and 112.0% in the first, third and fifth generation, respectively. Although the average production of positive mutants in the first generation was similar to four single mutageneses, the mean production of the positive mutants in the fifth generation was at least 3.9% higher than the single mutagenesis. Moreover, the number

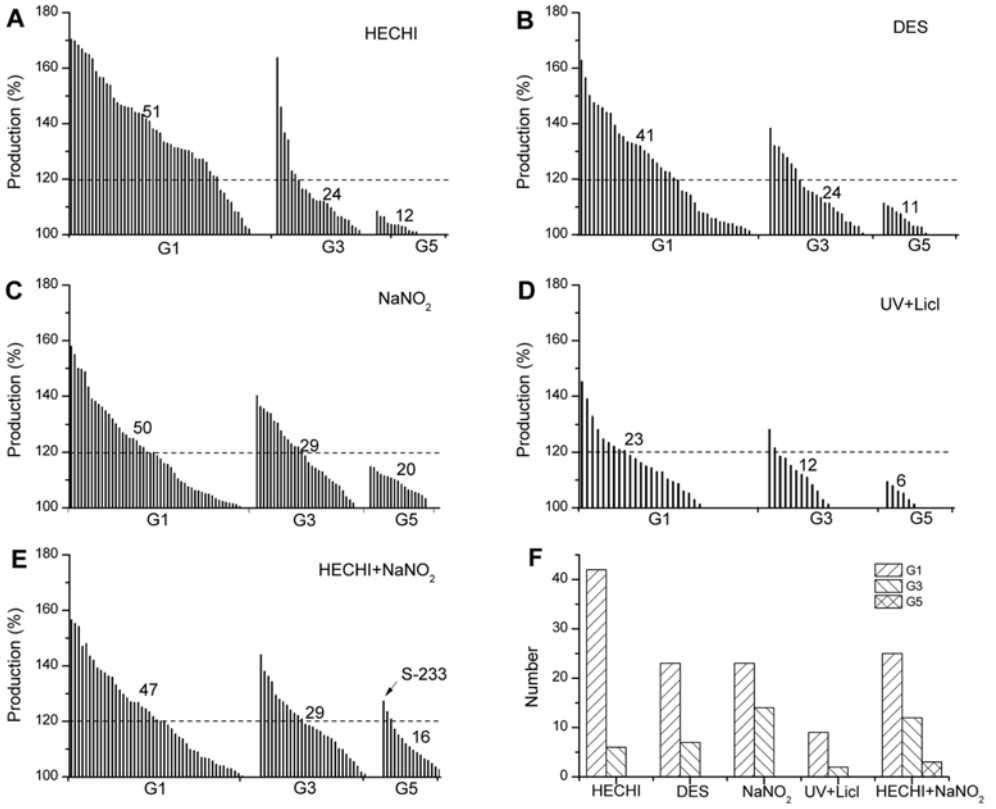
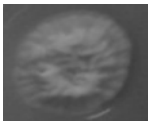
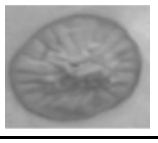


Fig. 3. Propagating stability of mutant strains treated by different mutation method. G1: the first generation, G3: the third generation, G5: the fifth generation, the number of positive strains was labelled. (A) HECHI, (B) diethyl sulfate, (C) sodium nitrite, (D) UV-irradiation combined with lithium chloride, (E) HECHI combined with sodium nitrite, (F) the number of mutants with production improved by more than 20% of different mutagenesis method.

Table 3. Morphology characteristics and positive mutant rate of compound mutation

Type	Photo	Colony morphology	Positive mutation rate (%)
1		Irregular in the middle	38.1%
2		Sagged in the middle	32.3%
3		Upward bulge in the middle	36.1%
4		Split in the middle	20.5%
5		Yellow in the middle	23.6%

of mutants with production improved by more than 20% was 25, 12, and 3 in the first, third and fifth generations, respectively (Fig. 3F). Three inherit-stable mutants with high avermectin B_{1a} production were successfully obtained. Results demonstrated that mutants treated with a combined mutagenesis exhibited better stability than any other single method. Additionally, the colonial morphology of the mutant strains was primarily classified into 5 types and their morphology characteristics and positive mutant rates were investigated in this study (Table 3). The strain, S-233, with the colonial morphology of type 2 and a production increase of 27.3% in the fifth generation, was screened out for avermectin B_{1a} production.

3.3. Verification of the selected mutant, *S. avermitilis* S-233, in a 500 L bioreactor based on dynamic physiological metabolism parameters

S-233, one of the high-yielding strains that has a production of 127.1% in a shake flask, was further validated in a 500 L bioreactor. As shown in Fig. 4, the prominent potential of S-233 was exhibited. There were obvious differences between the physiological characteristics of the S-233 and parent strains. During the growing period (0 ~ 48 h), the oxygen

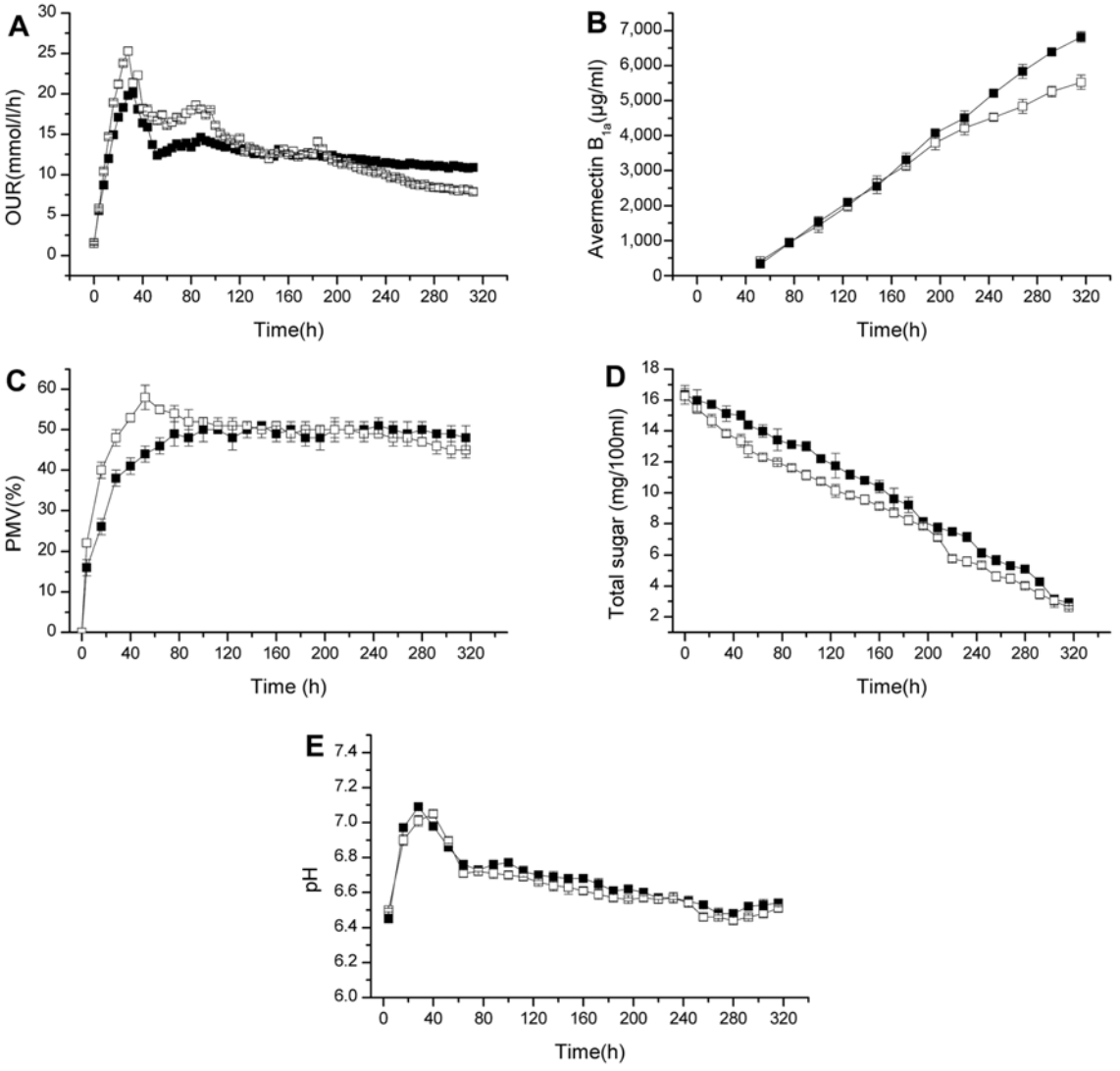


Fig. 4. The comparisons of fermentation parameters between parent strain (open square) and S-233 (filled square) in 500 L bioreactor.

uptake rate (OUR) and PMV of strain S-233 was lower than that of the control strain (Figs. 4A and 4 C). At the same time, the total sugar and pH of the strain-233 broth was higher than that of the control (Figs. 4D and 4E), which suggested that both the specific growth rate and metabolic intensity of S-233 were lower. During the production phase (48 ~ 280 h), the S-233 mutant strain showed a more stable OUR and PMV, increased total sugar consumption rate, slightly decreased pH, and an obviously higher production rate (Figs. 4A, 4B, 4C, 4D, and 4E), which indicated that the S-233 mutant strain, compared to its parent strain, exhibited a more stable level of metabolism and a stronger ability for avermectin B_{1a} synthesis. The slightly declined OUR and rebound pH in the late fermentation stage (*i.e.,* 280 ~ 312 h) might be due to a low total sugar content in the fermentation broth. Therefore, the mutant strain had a significant advantage compared with the original strain. Moreover, the production of avermectin B_{1a} was remarkably increased and reached 6818 μg/mL by the end of fermentation, which is 1.2-fold higher than that of the parent strain. However, the fermentation medium for S-233 called for constant optimization and study on the feeding processes that aimed to solve the lack of sugar in the late fermentation stage is ongoing in our laboratory.

DNA damage is generally recognized as the first step in the induction of mutation. High linear energy transfer (LET) radiation (*e.g.*, ion particles) causes localized, dense ionization within the cell compared to low-LET radiation (*e.g.,* X-rays or γ-rays) [24,25]. HECHI irradiation is a type of high linear energy transfer radiation and has been

effectively used due to its wide mutation spectrum and high ratio of mutation. HECHI is known to cause great damages to cellular components and particularly renowned for its ability to generate DNA double-strand breaks by irradiated cells locally [25,28]. Given the fact that heavy-ion beam was reported to result in more than 10 times the number of *Saccharomyces cerevisiae* mutations induced by γ-rays, the clustered double-strand breaks seemed to be more difficult to repair than single-stranded breaks caused by γ-rays [29-31]. In our study, the positive mutant strains from HECHI irradiation had the highest mean production and positive mutation rate in the first generation; however, the stability was not good in the third and fifth generations.

Typically, alternating physical and chemical mutagenesis provides a better mutagenesis frequency than a single round of mutation. In our study, we used HECHI irradiation and sodium nitrite sequentially as mutagens. Sodium nitrite has been generally recognized as a classical mutagen to make purine or pyrimidine deamination and change the structure of nucleic acids with GC→AT transitions [32]. The mutants from the combination of HECHI irradiation and sodium nitrite mutagenesis exhibited higher qualities than those of the single mutagenesis. The mean production of the positive mutants in the fifth generation was at least 3.9% higher than that of the single mutagenesis, and 3 inherit-stable mutants with high avermectin B_{1a} production were successfully obtained. Interestingly, alternating physical and chemical mutagenesis was also reported to have good effects. Li *et al.* enhanced arachidonic acid production with 40.61% from *Mortierella alpine* by combining atmospheric and room temperature plasma (ARTP) and diethyl sulfate treatments [27]. Additionally, *Saccharomyces cerevisiae* strains were mutagenized by means of a combined treatment of UV light, microwave and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) to improve the production of metallothionein. A high-activity strain was obtained with a total protein content and metallothionein content 170.2 mg/g cells and 163.4 ng/L, respectively; whereas, those of the parental strain were 44.6 mg/g cells and 38.3 ng/L, respectively [33]. Generally, the cell envelope was proposed to be the primary target of the physical radiation with effects ranging from the formation of pores to cell-wall ruptures. The formation of small pores leads to an entrance pathway for chemical mutagens into the cell [34,35]. We supposed that the permeability of the cell membrane increased after the treatment of HECHI, which would facilitate access of the sodium nitrite in the cell and strengthen the damages to DNA.

4. Conclusion

Although improvement strategies like metabolic engineering and directional genetic modification have been applied to improve the properties of the industrial strain in recent years [36-39], mutagenesis and selection still played a central role in the commercial development of microbial fermentation strains because gene manipulation was sometimes difficult or powerless [40,41]. In our study, a potent mutant, *S. avermitilis* S-233, with high avermectin B_{1a} production was successfully obtained. A pilot test proved that the S-233 strain possessed great potential for industry production, which indicated that HECHI irradiation combined with sodium nitrite was an effective method for the mutation breeding of avermectin B_{1a} production of *Streptomyces avermitilis*.

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