

## Research Article

# Significance of Heavy-Ion Beam Irradiation-Induced Avermectin B1a Production by Engineered *Streptomyces avermitilis*

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Heavy-ion irradiation technology has advantages over traditional methods of mutagenesis. Heavy-ion irradiation improves the mutation rate, broadens the mutation spectrum, and shortens the breeding cycle. However, few data are currently available regarding its effect on *Streptomyces avermitilis* morphology and productivity. In this study, the influence of heavy-ion irradiation on *S. avermitilis* when cultivated in approximately 10 L stirred-tank bioreactors was investigated. The specific productivity of the avermectin (AVM) B1a-producing mutant *S. avermitilis* 147-G<sub>5</sub>8 increased notably, from 3885 to 5446  $\mu\text{g}/\text{mL}$ , approximately 1.6-fold, compared to the original strain. The mycelial morphology of the mutant fermentation processes was microscopically examined. Additionally, protein and metabolite identification was performed by using SDS-PAGE, 2- and 3-dimensional electrophoresis (2DE and 3DE). The results showed that negative regulation gene deletion of mutants led to metabolic process upregulating expression of protein and improving the productivity of an avermectin B1a. The results showed that the heavy-ion beam irradiation dose that corresponded to optimal production was well over the standard dose, at approximately 80 Gy at 220 AMeV (depending on the strain). This study provides reliable data and a feasible method for increasing AVM productivity in industrial processes.

## 1. Introduction

Avermectins (AVMs) are metabolites from *Streptomyces avermitilis* [1]. This species' secondary metabolites have drawn great interest due to their unique structures and broad insecticide and anthelmintic activities [2–6]. The fermentation industry is attempting to improve the yield of AVM for future applications. Previous studies have shown that such products are positively affected by expression changes in housekeeping gene [7–10]. However, artificial manipulation of the genes involved in the AVM production does not readily lead to a mutant with satisfactory AVM B1a mass production. Furthermore, this genetic manipulation must be extremely precise, which would have high cost and potential for unsafe process operation. Some manipulations cannot be effectively scaled up for production in a fermentation plant. Although

$^{12}\text{C}^{6+}$  heavy-ion irradiation plays a key role in bioprocessing and biofermentation design and scale-up, no work has been done on heavy-ion irradiation and its effect on the growth and AVM productivity of *S. avermitilis*.

In this study, mutants with high productivity were used for process optimization with  $^{12}\text{C}^{6+}$  heavy-ion irradiation. The energy and dosage of  $^{12}\text{C}^{6+}$  heavy ions affected the phenotypes of survivors and generated mutants. Depending on the desired AVM or AVM B1a product, the original energy input and dose for a given bioprocess varied. Optimal key parameters, however, strongly correlated with the culture process and the operating parameters. To ensure the high production of the AVM B1a metabolite by mutants, it must be noted that the productivity of a bioprocess is not only determined by each specific process and its associated

parameters, but also significantly impacted by the dry cell weight, kinetics, agitation speed, the initial fair values ( $k_La$ ), dissolved oxygen (DO), and viscosity within the bioreactor [11–15]. The various process parameters and ingredients that influence the AVMs and AVM Bla production of the original strain have been extensively described in the literature [16–20]. Furthermore, many studies have shown that the DO, the oxygen supply, and the agitation speed are interrelated in varied culture conditions [21–23]; decoupling of these factors is often obtained by adjustment of the stirred-tank bioreactor stir speed, the type and number of stirred-tank bioreactors, the gas flow rate, cell growth, and the morphology of mycelia; metabolite biosynthesis variably depends on these factors [24–26]. Because the DO was found to be the primary factor affecting microbial metabolite production, most culture processes adopt a DO-control strategy by adjusting agitation. Future investigations should focus on identifying the essential regulating factors in the culture process [27, 28], which have vital importance for achieving high cultivation performance.

In the present work, heavy-ion irradiation and its effect on the growth and specific productivity of *S. avermitilis* were studied. The effects of the energy and dose of  $^{12}\text{C}^{6+}$  heavy ions were determined and then these parameters have been used to develop a customized bioreactor and bioprocess design. These findings may prove to be invaluable and helpful in industrial design and scale-up or for other applications.

## 2. Materials and Methods

**2.1. Experimental Setup and Heavy-Ion Beam Irradiation.** Heavy-ion beam experimental setups were employed as previously described [29]. The extraction time of the  $^{12}\text{C}^{6+}$  heavy ions (approximately 140 AMeV, 180 AMeV, and 220 AMeV of energy) was approximately 3 s, the priming dose was 80 Gy, and the dose rates were up to 10 Gy/min. In this study, the operating parameters were as follows: the radiation energy input was 140, 180, and 220 AMeV; the temperature of the  $^{12}\text{C}^{6+}$  heavy-ion beams was  $<35^\circ\text{C}$  under these conditions [30, 31].

**2.2. Strain and Culture Medium.** The original *S. avermitilis* strain (AV-J-AO) was obtained from the Industrial Microbial Culture Collection Center of Gansu Province, China. The original culture medium consisted of the followings:  $\text{KNO}_3$  1.5 g/L,  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  0.5 g/L,  $\text{NaCl}$  0.5 g/L,  $\text{FeSO}_4$  0.01 g/L, corn starch 25 g/L, yeast extract 2.0 g/L, and soluble agar 20 g/L in distilled water at  $\text{pH } 7.3 \pm 0.1$ . The original seeding medium for mutant strains consisted of the followings: corn starch 40 g/L, yeast extract 5 g/L, soy flour 3.5 g/L, and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.02 g/L in distilled water at  $\text{pH } 7.5 \pm 0.1$ . The original fermentation medium consisted of the following:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.6 g/L,  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  0.6 g/L,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.02 g/L,  $\text{CaCO}_3$  2.5 g/L, and  $\text{KCl}$  5 g/L in distilled water at  $\text{pH } 7.5 \pm 0.1$ . All media were autoclaved at  $121^\circ\text{C}$  for 20 min.

**2.3. Experimental Protocol for Mutant Strains.** The  $^{12}\text{C}^{6+}$  heavy-ion irradiated spore solution was spread on the original seeding of mutant strains medium and cultivated to

form colonies. After incubation for 6 days at  $30^\circ\text{C}$ , many single colonies with various morphologies were observed. Each colony was counted and isolated. A few isolates were selected as inoculants for fermentation at  $30^\circ\text{C}$  for 12 days to examine the specific productivity of AVM Bla.

**2.4. Bioreactor Configuration.** The geometric parameters of the bioreactor are as follows: diameter ( $T$ ), 220 mm; liquid height ( $H$ ), 89 mm; bottom type, flat; filled volume ( $V_L$ ), 7 L; capacity ( $V$ ), 10 L; impellers, shear. The main cultivation conditions were as follows: temperature,  $28^\circ\text{C}$ ; air flow rate, 50 L/min; stirring speed, 180–200 rpm beginning 3 h after inoculation. The DO was controlled above 20% in all fermentation processes.

**2.5. Detection of the Lethality and Mutation Rates.** After irradiating the inoculated plates, the lethality and mutation rates were calculated using the following equations:

$$\text{Survival rate} = \left[ \frac{T}{U} \right] \times 100\%$$

$$\text{Positive mutation rate } (R_{M/T}) = \left[ \frac{M}{T} \right] \times 100\% \quad (1)$$

$$\text{Negative mutation rate } (R_{P/T}) = \left[ \frac{P}{T} \right] \times 100\%,$$

where  $U$  is the total number of colonies of the sample without treatment;  $T$  is the total number of colonies after treatment with plasma;  $M$  is the number of colonies of the mutant strains that produce less AVM Bla than the original strain; and  $P$  is the number of colonies of mutants that produce more AVM Bla than the original strain.

**2.6. Mycelial Soluble Protein Extraction and Analysis.** Intracellular protein extracts were prepared for analysis by 2- and 3DE based on the method of Jun et al. [32]. Proteins were visualized using Coomassie brilliant blue staining as described by Neuhoff et al. [33]. Spots of interest were excised, and in-gel digestion with trypsin was performed as described by Jun et al. [32].

**2.7. Measurement of Cell Growth, Residual Dextrin, and AVM and AVM Bla Production.** Three milliliters of culture broth was taken for each time point and centrifuged at 3,500 rpm for 10 min. The pellet was dried to constant weight at  $110^\circ\text{C}$  to measure the dry cell weight (DCW). The total dextrin consumption was determined using dextrin assay kit according to the manufacturer's instructions (Rongsheng Biotech. Ltd., Shanghai). AVM and AVM Bla were analyzed by HPLC (LC10A; Shimadzu, Japan).

**2.8. Measurement of Fair Value.** The initial fair values ( $k_La$ ) in bioreactors were prepared for analysis by the non-steady-state method [34] and were calculated by the following equation:

$$\frac{dc}{dt} = k_La(C^* - C), \quad (2)$$

where  $dc/dt$  represents the DO change over time,  $C^*$  denotes the saturated DO concentration, and  $C$  denotes the DO at a specific time point. The  $k_L a$  value ( $\text{h}^{-1}$ ) was calculated as the slope of  $\ln(C^* - C)$  versus time.

**2.9. Determination of Agitation Speed.** Determination of the agitation speed was calculated by the following equation (3):

$$V = \frac{\pi \times d \times N}{1,000}, \quad (3)$$

where  $d$  is the turbine diameter (0.055 m here) and  $N$  is the agitation speed (runs per second).

**2.10. Calculation of Dry Cell Weight (DCW).** The DCW was calculated with the following equations (4):

$$\text{DCW} = \int_{t_1}^{t_2} \text{DCW} \cdot dt \quad (4)$$

$$\text{DCW}_{t_2} = \text{DCW}_{t_1} + \left[ \frac{\text{DCW}_{t_1} + \text{DCW}_{t_2}}{2} \cdot (t_2 - t_1) \right],$$

where  $\text{DCW}_{t_1}$  is the first point during the fastest logarithmic growth period and  $\text{DCW}_{t_2}$  is the last point.  $t_1$  and  $t_2$  are similarly described.

**2.11. Determination of Oxygen Uptake Rate (OUR) and Specific Oxygen Uptake Rate (SOUR).** The oxygen consumption rate was determined as described by Garcia-Ochoa and Gomez [27]. The SOUR was calculated as the OUR was divided by the DCW at each time point.

**2.12. Determination of Broth Viscosity and Cell Morphology.** Broth viscosity was detected as described by Kato et al. [35]. At each sampling point, the morphology of samples placed on a slide was observed with a Nikon microscope (Nikon Eclipse TE2000-U, Tokyo, Japan).

**2.13. Examination of Mutant Genetic Stability.** For the first subculture, the mutant strains were streaked or spread and cultivated on medium for 6 days. For the second subculture, several single colonies were selected and streaked onto new solid medium plates for second 6-day cultivation. The same procedure was repeated for a total of six subcultures. After each subculture, the specific productivity of AVMs and AVM Bla was evaluated by fermentation.

**2.14. Statistical Analysis.** All data represent the result of three independent samples (flasks). The error bars indicate the standard deviation (SD) from the mean of experiments performed in triplicate. The data were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. A two-tailed  $p$  value  $< 0.05$  was considered statistically significant. SPSS 18.0 software for Windows was used for the statistical analyses (SPSS Inc. Chicago, IL, USA).

### 3. Results

**3.1. Independent  $^{12}\text{C}^{6+}$  Heavy-Ion Irradiation Experiments.** The ideal working protocol for the treatment of *S. avermitilis* spores by exposure to  $^{12}\text{C}^{6+}$  heavy-ion irradiation was explored. Based on the strain characteristics, the effect of heavy-ion beam irradiation on survival was evaluated for independent irradiation experiments. A number of post irradiation survivors after irradiation used a  $^{12}\text{C}^{6+}$  heavy-ion beam with an energy input of 140 AMeV at dose of 80 Gy at a dose rate of 10 Gy/min. The survival rate of the spores treated with 80 Gy was 13.7%; a very limited survival (8.4%) was obtained with 80 Gy of 180 AMeV  $^{12}\text{C}^{6+}$  heavy-ion irradiation. For further confirmation of these results, we increased the energy input to 220 AMeV at a dose of 80 Gy. There was only 3.4% survival under these conditions. Collectively, the data of survival rate for spores exposed to carbon ion beams of different energies are questionable. With increasing the energy of carbon ion beam, the value of linear energy transfer (LET) decreases. So the survival rate of spores should increase when increasing the beam energy. However, it is interesting that the data presented in our study showed an opposite tendency. Obviously, a lower energy of carbon ion beam is credited with a higher the value of LET, but the beam range is very short. It is for this reason that  $^{12}\text{C}^{6+}$  heavy-ion irradiation is the dose of radiation hot spot and cold spot effect, namely, some of the *S. avermitilis* spores were not irradiated or are exposed to very small doses of radiation.

**3.2. Strain Morphology before and after  $^{12}\text{C}^{6+}$  Heavy-Ion Treatment at Different Energy Inputs and Dosages.** After exposure to a high dose of high-energy  $^{12}\text{C}^{6+}$  heavy ions, the survivors may represent mutants. The survival rates of 13.7%, 8.4%, and 3.4% were determined after cultivation on medium for 6 days at 28°C. In total, there were more than 1000 single colonies growing on 100 plates. These colonies were clearly different from those that developed from nonirradiated spores. The survivors displayed multiple colony texture and colors, such as the flat straw, grey steamed bread, and white bald and taupe crimple forms. The colonies were cultivated from survivors of  $^{12}\text{C}^{6+}$  heavy-ion irradiation with energy inputs of 140, 180, and 220 AMeV at doses of 80 Gy, respectively. These sample points were randomly selected based on the survival rates before any data processing tasks were undertaken to avoid bias from human operators. The colonies from each of the 140-, 180-, and 220-AMeV treatments were classified into 8 groups, namely, AO-M<sub>3</sub>1 to AO-M<sub>3</sub>8, HS-P<sub>1</sub>1 to HS-P<sub>1</sub>8, and 147-G<sub>5</sub>1 to 147-G<sub>5</sub>8, respectively; the corresponding data are shown in Tables 1, 2, and 3, respectively.

**3.3. Specific Production of AVMs and AVM Bla.** The AVM and AVM Bla specific productivity of the nonirradiated original strains and the 24 selected mutant strains was determined by fermentation experiments in approximately 1 L stirred-tank bioreactors. For the nonirradiated original strain *S. avermitilis*, the yield values of AVM Bla and total AVMs,  $Y_{\text{AVM Bla}}$ , and  $Y_{\text{Total AVMs}}$  were  $3885 \pm 180$  and  $4530 \pm 270$   $\mu\text{g}/\text{mL}$ , respectively, based on 3 replicates. Table 1 shows

TABLE 1: Comparison of strain-specific productivity after  $^{12}\text{C}^{6+}$  heavy-ion irradiation with energy input of 140 AMeV at a dose of 80 Gy ( $n = 5$ ).

Group	Number of colonies	Strain	$Y_{\text{AVM B1a}}^*$	$Y_{\text{Total AVMs}}^*$	$\gamma$ (%)**
		The original strain AV-J-AO	$1.00 \pm 0.11$	$1.00 \pm 0.09$	40.3
AO-M <sub>3</sub> 1	8	AO-M <sub>3</sub> 1-1	$1.44 \pm 0.25$	$1.24 \pm 0.15$	47.1
		AO-M <sub>3</sub> 1-2	$1.52 \pm 0.13$	$1.27 \pm 0.18$	51.4
AO-M <sub>3</sub> 2	13	AO-M <sub>3</sub> 2-1	$1.15 \pm 0.22$	$1.12 \pm 0.26$	40.9
		AO-M <sub>3</sub> 2-2	$1.18 \pm 0.17$	$0.94 \pm 0.12$	42.5
<i>P</i>	21				
AO-M <sub>3</sub> 3	17	AO-M <sub>3</sub> 3-1	$0.78 \pm 0.03$	$0.36 \pm 0.09$	58.9
		AO-M <sub>3</sub> 3-2	$0.42 \pm 0.07$	$0.58 \pm 0.06$	26.7
AO-M <sub>3</sub> 4	16	AO-M <sub>3</sub> 4-1	$0.36 \pm 0.06$	$0.42 \pm 0.08$	53.7
		AO-M <sub>3</sub> 4-2	$0.57 \pm 0.02$	$0.51 \pm 0.06$	51.4
AO-M <sub>3</sub> 5	27	AO-M <sub>3</sub> 5-1	$0.43 \pm 0.07$	$0.40 \pm 0.03$	49.8
		AO-M <sub>3</sub> 5-2	$0.37 \pm 0.04$	$0.43 \pm 0.07$	53.3
AO-M <sub>3</sub> 6	8	AO-M <sub>3</sub> 6-1	$0.94 \pm 0.02$	$0.97 \pm 0.04$	48.9
		AO-M <sub>3</sub> 6-2	$0.47 \pm 0.04$	$0.32 \pm 0.09$	45.7
AO-M <sub>3</sub> 7	9	AO-M <sub>3</sub> 7-1	$0.27 \pm 0.03$	$0.17 \pm 0.02$	31.5
		AO-M <sub>3</sub> 7-2	$0.17 \pm 0.05$	$0.07 \pm 0.06$	—
<i>M</i>	77				
AO-M <sub>3</sub> 8	328	AO-M <sub>3</sub> 8-1	$1.07 \pm 0.03$	$1.16 \pm 0.05$	63.2
		AO-M <sub>3</sub> 8-2	$0.93 \pm 0.03$	$0.87 \pm 0.11$	38.3
<i>T</i>	426				
$R_{M/T} = M/T = 18.1\%$			$R_{P/T} = P/T = 4.9\%$		

\* $Y_{\text{AVM B1a}}$  and  $Y_{\text{Total AVM}}$  represent the percentage content of specific productivity of all mutant's  $Y_{\text{AVMs B1a}}$  and  $Y_{\text{Total AVMs}}$  to those of the original strain *S. avermitilis*, respectively. \*\* $\gamma$  (%) indicates the ratio of  $Y_{\text{AVMs B1a}}$  to  $Y_{\text{Total AVMs}}$ .

the specific AVM productivity of strains irradiated with  $^{12}\text{C}^{6+}$  heavy-ion beam with an energy input of 140 AMeV at a dose of 80 Gy as a percentage of the original strain productivity. Among the randomly selected and examined strains, AO-M<sub>3</sub>1 showed the highest  $Y_{\text{AVM B1a}}$ , which was over 1.48-fold higher than that of the nonirradiated original strain. Moreover, the  $Y_{\text{Total AVMs}}$  of AO-M<sub>3</sub>1 also increased by approximately 1.24-fold compared to that of nonirradiated original strain. Two other mutant strains, AO-M<sub>3</sub>2 and AO-M<sub>3</sub>8, showed 1.03-fold and 1.06-fold higher AVM specific productivity than the original strain, respectively. Table 2 shows the AVM specific productivity of the strains that were irradiated with  $^{12}\text{C}^{6+}$  heavy-ion beam at an energy input of 180 AMeV and a dose of 80 Gy relative to the original strain. Among the randomly selected and examined strains, HS-P<sub>1</sub>8 showed the highest  $Y_{\text{AVM B1a}}$ , which was over 1.44-fold higher than that of the original strain. Table 3 shows the AVM specific productivity of the strains irradiated with  $^{12}\text{C}^{6+}$  heavy ions at an energy input of 220 AMeV and a dose of 80 Gy relative to the original strain. Among the randomly selected and examined strains, 147-G<sub>5</sub>8 shows the highest  $Y_{\text{AVM B1a}}$ , which was over 1.76-fold higher than that of the original strain.

**3.4. The Mutation Rate and Positive Mutation Rate.** Tables 1, 2, and 3 show the ratios of  $Y_{\text{AVM B1a}}$  to  $Y_{\text{Total AVM}}$  ( $\gamma$ ) for all of

the mutants and for original strain *S. avermitilis*. The value of a metabolite production for the original strain was 22%, 25%, and 41%; for most mutants, it changed by more than 15%. The specific productivity of the *S. avermitilis* mutants in this determination had approximately 5% random error, based on the average data from quintuplicate measurements. Thus, only strains with greater than 5% increases or decreases in  $Y_{\text{AVM B1a}}$  or  $Y_{\text{Total AVMs}}$  compared to the original strain were identified as “Positive” or “Negative” mutants, respectively. Based on the specific productivity of 24 strains and the colony number of each group, the positive mutation rate  $R_{M/T}$  was estimated as 18.1%, 24.6%, and 22.8% for the 140-, 180-, and 220-AMeV treatments, respectively. The negative mutation rate  $R_{P/T}$  was estimated to be 4.9%, 12.7%, and 12.3% for the 140-, 180-, and 220-AMeV treatments, respectively, as shown in Tables 1, 2, and 3, respectively.

**3.5. Kinetics of Original Strain and AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 Growth in Shake Flasks.** Figure 1 shows the kinetic growth profiles of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant strains and the original strain. The biomasses of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants and the original strain began to increase after inoculation, reached maxima at 72 h, and then reached platform stage of growth. As shown in Figure 1(a), the growth of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and



TABLE 2: Comparison of strain-specific productivity after  $^{12}\text{C}^{6+}$  heavy-ion irradiation with energy input of 180 AMeV at a dose of 80 Gy ( $n = 5$ ).

Group	Number of colonies	Strain	$Y_{\text{AVM Bla}}^*$	$Y_{\text{Total AVMs}}^*$	$\gamma$ (%)**
		The original strain AV-J-AO	$1.00 \pm 0.11$	$1.00 \pm 0.09$	40.3
HS-P <sub>1</sub> 1	31	HS-P <sub>1</sub> 1-1	$1.34 \pm 0.35$	$1.32 \pm 0.17$	52.4
		HS-P <sub>1</sub> 1-2	$1.32 \pm 0.18$	$1.19 \pm 0.15$	46.3
HS-P <sub>1</sub> 2	23	HS-P <sub>1</sub> 2-1	$1.09 \pm 0.22$	$0.97 \pm 0.31$	37.5
		HS-P <sub>1</sub> 2-2	$1.26 \pm 0.22$	$1.12 \pm 0.19$	39.7
<i>P</i>	54				
HS-P <sub>1</sub> 3	21	HS-P <sub>1</sub> 3-1	$0.26 \pm 0.02$	$0.15 \pm 0.07$	25.9
		HS-P <sub>1</sub> 3-2	$0.38 \pm 0.04$	$0.45 \pm 0.03$	37.1
HS-P <sub>1</sub> 4	25	HS-P <sub>1</sub> 4-1	$0.16 \pm 0.01$	$0.05 \pm 0.02$	—
		HS-P <sub>1</sub> 4-2	$0.27 \pm 0.04$	$0.31 \pm 0.03$	27.3
HS-P <sub>1</sub> 5	31	HS-P <sub>1</sub> 5-1	$0.56 \pm 0.03$	$0.43 \pm 0.05$	35.6
		HS-P <sub>1</sub> 5-2	$0.42 \pm 0.04$	$0.57 \pm 0.12$	49.3
HS-P <sub>1</sub> 6	11	HS-P <sub>1</sub> 6-1	$1.13 \pm 0.06$	$0.83 \pm 0.09$	39.8
		HS-P <sub>1</sub> 6-2	$0.12 \pm 0.04$	$0.08 \pm 0.09$	—
HS-P <sub>1</sub> 7	17	HS-P <sub>1</sub> 7-1	$0.34 \pm 0.03$	$0.21 \pm 0.04$	39.1
		HS-P <sub>1</sub> 7-2	$0.59 \pm 0.05$	$0.47 \pm 0.06$	43.8
<i>M</i>	105				
HS-P <sub>1</sub> 8	267	HS-P <sub>1</sub> 8-1	$1.54 \pm 0.09$	$0.97 \pm 0.03$	67.3
		HS-P <sub>1</sub> 8-2	$1.34 \pm 0.11$	$1.05 \pm 0.15$	43.9
<i>T</i>	426				
$R_{M/T} = M/T = 24.6\%$			$R_{P/T} = P/T = 12.7\%$		

\* $Y_{\text{AVM Bla}}$  and  $Y_{\text{Total AVM}}$  represent the percentage content of specific productivity of all mutant's  $Y_{\text{AVMs Bla}}$  and  $Y_{\text{Total AVMs}}$  to those of the original strain *S. avermitilis*, respectively. \*\* $\gamma$  (%) indicates the ratio of  $Y_{\text{AVMs Bla}}$  to  $Y_{\text{Total AVMs}}$ .

147-G<sub>5</sub>8 mutants was much faster than that of the original type. The 147-G<sub>5</sub>8 mutant's maximum DCW was  $13.7 \pm 1.5$  g/L, which was significantly higher than the  $12.7 \pm 0.7$  g/L of the original strain. Dextrin consumption was in accord with each strain's cell growth; the sugar was consumed over 69.2% after 120 h by these mutants. This phenomenon may be explained as the higher biomass increase of mutants after more than 120 h compared to the original strain. As shown in Figure 1(b), the AVM Bla concentration continued to increase throughout the culture process of the mutants, while almost no AVM Bla was detected for the original strain. The maximal AVM Bla production by the 147-G<sub>5</sub>8 mutant was  $1575 \pm 120$   $\mu\text{g}/\text{mL}$  at 120 h in convention shake flask culture. The results demonstrate the feasibility of AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant cultivation for producing a certain amount of AVM Bla, and the 147-G<sub>5</sub>8 mutant showed the best specific productivity.

**3.6. Effects of Shaking Speed and Filling Volume on AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 Mutant Growth in Flasks.** AVM Bla production was tested at different shaking speeds and filling volumes in baffled shake flasks. Shaking speed was the most important factor affecting AVM Bla production. At 250 rpm, the AVM Bla production of the AO-M<sub>3</sub>1 mutant ranged from 3889 to 3979  $\mu\text{g}/\text{mL}$  with filling volumes ranging from 20

to 80 mL. For the HS-P<sub>1</sub>8 mutant, production ranged from 4178 to 4238  $\mu\text{g}/\text{mL}$  with filling volumes ranging from 10 to 90 mL, and for the 147-G<sub>5</sub>8 mutant they ranged from 4560 to 4770  $\mu\text{g}/\text{mL}$  with filling volumes ranging from 10 to 100 mL. At 200 rpm, the AVM Bla production was only 3889–3927  $\mu\text{g}/\text{mL}$  for the AO-M<sub>3</sub>1 mutant, 4178–4216  $\mu\text{g}/\text{mL}$  for HS-P<sub>1</sub>8, and 4560–4630  $\mu\text{g}/\text{mL}$  for 147-G<sub>5</sub>8. The productivity of three mutants increased about 38~70  $\mu\text{g}/\text{mL}$  when the shaking speed increased from 200 to 250 rpm, but it varied only marginally with different filling volumes at a given shaking speed. The production of AVM Bla and total AVMs by the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants was greater than that by the original strain. Figure 2 shows that the 147-G<sub>5</sub>8 cellular morphology became much more hairy, with many thick hyphae, in contrast to the original strain, in which the majority of hyphae formed large, dense, and uniform pellets. After 256 h of cultivation in shake flasks, the original strain exhibited the hyphal autolysis obvious phenomenon.

**3.7. Identification of the Most Highly Expressed Soluble Proteins in the Original Strain, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 Mutants.** A 2DE technique was used to identify the protein with the highest expression in the proteome of each mutant. Figure 3(a) shows 2DE of the whole-protein fractions of the original strain, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants. The range of protein separation

TABLE 3: Comparison of strain-specific productivity after  $^{12}\text{C}^{6+}$  heavy-ion irradiation with energy input of 220 AMeV at a dose of 80 Gy ( $n = 5$ ).

Group	Number of colonies	Strain	$Y_{\text{AVM B1a}}^*$	$Y_{\text{Total AVMs}}^*$	$\gamma$ (%)**
		The original strain AV-J-AO	$1.00 \pm 0.11$	$1.00 \pm 0.09$	40.3
147-G <sub>5</sub> 1	18	147-G <sub>5</sub> 1-1	$1.61 \pm 0.13$	$1.21 \pm 0.15$	61.5
		147-G <sub>5</sub> 1-2	$1.44 \pm 0.09$	$1.31 \pm 0.18$	42.9
147-G <sub>5</sub> 2	23	147-G <sub>5</sub> 2-1	$1.71 \pm 0.22$	$1.27 \pm 0.26$	62.4
		147-G <sub>5</sub> 2-2	$1.43 \pm 0.13$	$1.12 \pm 0.07$	63.7
<i>P</i>	41				
147-G <sub>5</sub> 3	13	147-G <sub>5</sub> 3-1	$1.14 \pm 0.03$	$0.78 \pm 0.09$	57.3
		147-G <sub>5</sub> 3-2	$1.32 \pm 0.06$	$0.69 \pm 0.11$	48.8
147-G <sub>5</sub> 4	18	147-G <sub>5</sub> 4-1	$1.25 \pm 0.05$	$0.52 \pm 0.07$	50.1
		147-G <sub>5</sub> 4-2	$1.07 \pm 0.02$	$0.61 \pm 0.06$	49.3
147-G <sub>5</sub> 5	21	147-G <sub>5</sub> 5-1	$0.38 \pm 0.03$	$0.07 \pm 0.03$	—
		147-G <sub>5</sub> 5-2	$0.96 \pm 0.07$	$0.72 \pm 0.02$	37.3
147-G <sub>5</sub> 6	11	147-G <sub>5</sub> 6-1	$0.87 \pm 0.02$	$0.97 \pm 0.04$	32.7
		147-G <sub>5</sub> 6-2	$1.07 \pm 0.11$	$0.43 \pm 0.13$	48.9
147-G <sub>5</sub> 7	13	147-G <sub>5</sub> 7-1	$0.45 \pm 0.03$	$0.27 \pm 0.02$	43.4
		147-G <sub>5</sub> 7-2	$0.13 \pm 0.02$	$0.05 \pm 0.01$	—
<i>M</i>	76				
147-G <sub>5</sub> 8	217	147-G <sub>5</sub> 8-1	$1.87 \pm 0.03$	$1.26 \pm 0.05$	69.2
		147-G <sub>5</sub> 8-2	$1.65 \pm 0.09$	$1.42 \pm 0.12$	61.4
<i>T</i>	334				
$R_{M/T} = M/T = 22.8\%$			$R_{P/T} = P/T = 12.3\%$		

\* $Y_{\text{AVM B1a}}$  and  $Y_{\text{Total AVM}}$  represent the percentage content of specific productivity of all mutant's  $Y_{\text{AVMs B1a}}$  and  $Y_{\text{Total AVMs}}$  to those of the original strain *S. avermitilis*, respectively. \*\* $\gamma$  (%) indicates the ratio of  $Y_{\text{AVMs B1a}}$  to  $Y_{\text{Total AVMs}}$ .

obtained by 2DE was 24–97 kDa and 4–7 pI. 2DE of the original strain, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants yielded 345, 418, and 591 protein spots, respectively. All of these proteins were also presented in samples that were analyzed by PD-Quest. Comparing to original strain, the 147-G<sub>5</sub>8 had 3-fold higher concentrations with 29 147-G<sub>5</sub>8 protein spots. HS-P<sub>1</sub>8 had 29 protein spots with 2-fold higher concentrations than original strain. Among them, comparisons in the ranges of 29–40.5 kDa and 4.2–5.1 pI are notable differences. Figure 3(b) shows that the 147-G<sub>5</sub>8 mutant had the highest overall expression and HS-P<sub>1</sub>8 the least, with weak overall expression observed. Figure 3(c) also shows that the expression of the 147-G<sub>5</sub>8 mutant was higher than that of original strain, although expression was only weakly observed. These results demonstrate the feasibility of cultivating the 147-G<sub>5</sub>8 mutant to produce certain amount of AVMs and metabolites.

**3.8. Impact of Agitation Speed on the Physiological Status of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 Mutants When Cultivated in Stirred-Tank Bioreactors.** To investigate the effect of agitation intensity on the cultivation of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants in stirred-tank bioreactors, the cell growth, residual dextrin, AVM B1a yields, DO, OUR, SOUR, and broth viscosity at agitation speeds of 150, 250, and 350 rpm

and at a fixed aeration rate of 1.0 vvm were examined in this study. As shown in Figure 4(a), the highest biomass production by the 147-G<sub>5</sub>8 mutant was 13.8 g/L at 150 rpm; this value slightly decreased at 250 rpm and dramatically dropped at 350 rpm, indicating a detrimental impact of a 350 rpm agitation speed on growth. This pattern was also observed for the AO-M<sub>3</sub>1 and HS-P<sub>1</sub>8 mutants (data not shown). As shown in Figure 4(b), dextrin consumption by the 147-G<sub>5</sub>8 mutant was similar at different agitation speeds and was completed at 96 h, after the biomass productivity reached its maximum. The pattern of consumption was similar for the AO-M<sub>3</sub>1 and HS-P<sub>1</sub>8 mutants (data not shown). As shown in Figure 4(c), AVM B1a production by the 147-G<sub>5</sub>8 mutant was higher at a relatively lower agitation speed; 896  $\mu\text{g}/\text{mL}$  was obtained at 150 rpm, 816  $\mu\text{g}/\text{mL}$  was obtained at 250 rpm, and 738  $\mu\text{g}/\text{mL}$  was obtained at 350 rpm, which was approximately 21.4% lower than what was achieved at 150 rpm. When the agitation speed was increased to 400 rpm, the production of AVM B1a by the 147-G<sub>5</sub>8 mutant decreased dramatically to 473  $\mu\text{g}/\text{mL}$ ; this phenomenon was also exhibited by the AO-M<sub>3</sub>1 and HS-P<sub>1</sub>8 mutants (data not shown). It would be interesting to determine the detrimental effect of an agitation speed greater than 350 rpm on the cellular physiology and metabolism of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant strains. The 147-G<sub>5</sub>8 mutant DO,

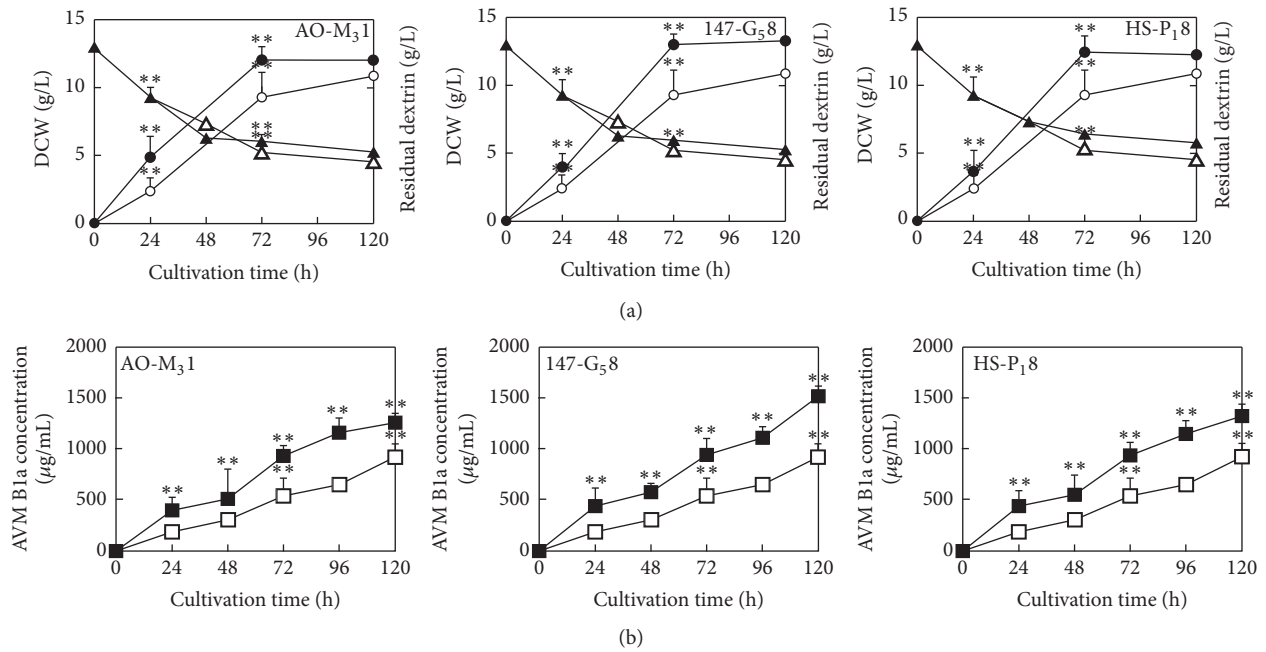


FIGURE 1: Growth kinetics of the original strain and the mutant strains AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 cultivated with shaking. (a) Time course of the cell dry weight and dextrin consumption of the original strain and the mutant strains AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8. Left panel, comparison of the AO-M<sub>3</sub>1 mutant (black circles and black triangles) with original strain *S. avermitilis* (white circles and white triangles). Middle panel, comparison of the 147-G<sub>5</sub>8 mutant (black circles and black triangles) with original strain *S. avermitilis* (white circles and white triangles). Right panel, comparison of the HS-P<sub>1</sub>8 mutant (black circles and black triangles) with original strain *S. avermitilis* (white circles and white triangles). (b) Time profiles of the AVM B1a specific productivity of original strain and AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant. On the left is comparison of AO-M<sub>3</sub>1 mutant (black box) with the original strain *S. avermitilis* (white box), the comparison of 147-G<sub>5</sub>8 mutant (black box) with the original strains *S. avermitilis* (white box) is in the middle, and on the right is comparison of HS-P<sub>1</sub>8 mutant (black box) with the original strains *S. avermitilis* (white box). Data are the average of quintuplicate samples; the error bars indicate the standard deviations. All results were analyzed with Tukey's test (duplicate determinations, \*\*  $p < 0.01$ ).

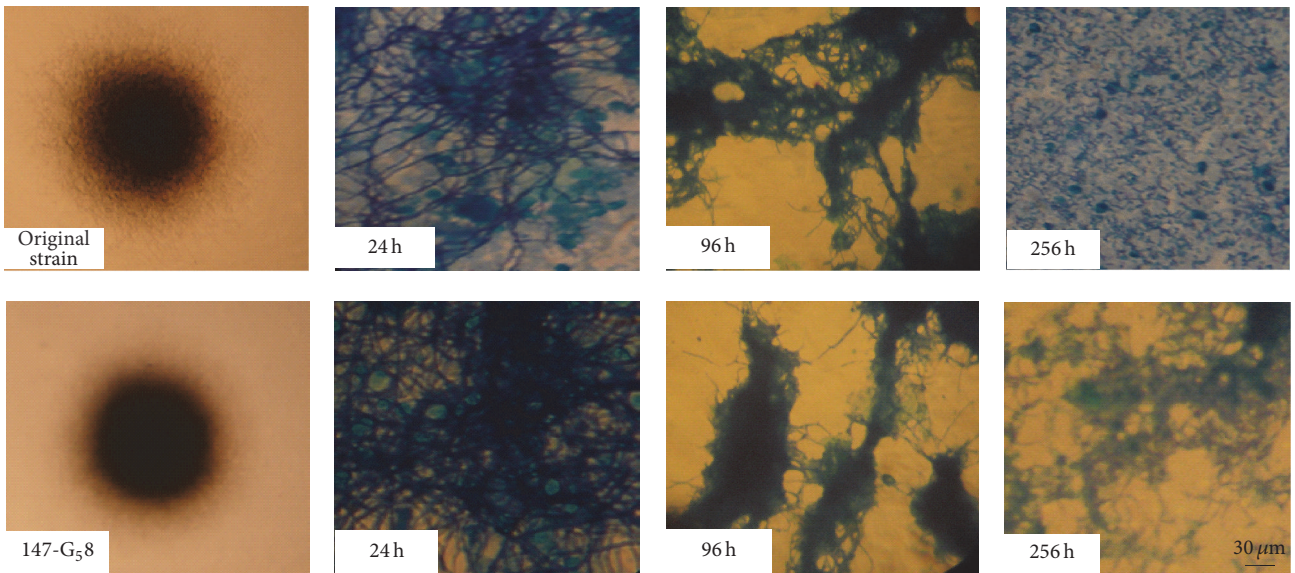


FIGURE 2: Comparison of the mycelial morphology between original strain *avermitilis* and the 147-G<sub>5</sub>8 mutant with optimized agitation at 250 rpm at different time points.



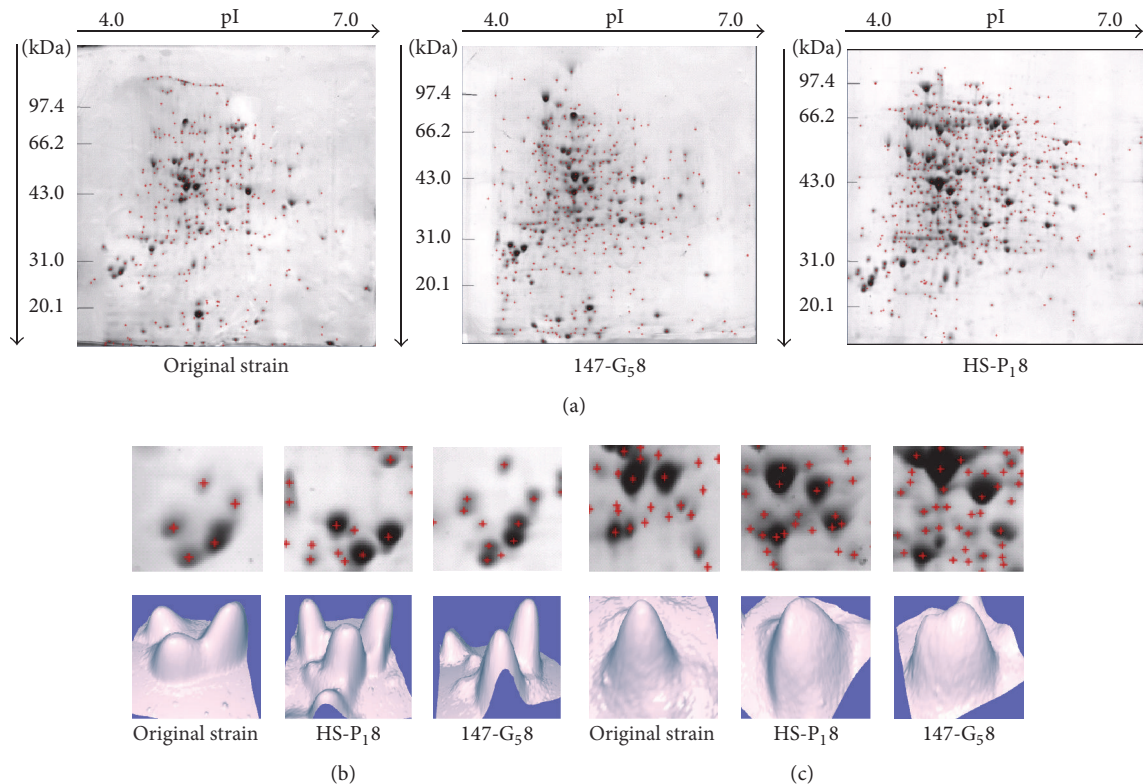


FIGURE 3: 2DE and 3DE gels of soluble proteins to determine the most highly expressed proteins of the *S. avermitilis* original strain, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants. (a) The soluble proteins with the highest expression levels in original strain, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants were identified on 2DE gels. (b) The 3 strains were analyzed by 3DE in the ranges of 29 kDa and 4.2 pI. (c) The 3 strains were analyzed by 3DE in the ranges of 40.5 kDa and 5.1 pI. The figure shows that the expression of the 147-G<sub>5</sub>8 mutant was higher than that of original strain, although expression was only weakly observed.

OUR, and SOUR were recorded over time, as shown in Figures 4(d), 4(e), and 4(f), respectively. The dotted, dashed line representing DO remained above 25% throughout the culture process in stirred-tank bioreactors, guaranteeing a sufficient oxygen supply. The highest OUR and SOUR values, 7.3 mmol/L/h and 0.7 mmol/g/h, respectively, occurred at 42 h. Overall, a higher agitation speed resulted in lower OUR and SOUR values for the mutants, indicating weak respiration activity, which was in agreement with the rapid cell growth, lower biomass accumulation, and AVM Bla formation. The exception was an agitation speed of 350 rpm, at which the AO-M<sub>3</sub>1 (data not shown), HS-P<sub>1</sub>8 (data not shown), and 147-G<sub>5</sub>8 mutants started to rapidly synthesize AVM Bla and showed an abnormally high OUR. The broth viscosity was over time during culture of the 147-G<sub>5</sub>8 mutant in stirred-tank bioreactors. The viscosity reached its maximum at 72 h in all experimental conditions before decreasing, following a similar pattern as AVM Bla production. It should be noted that, at 350 rpm, the higher agitation speed caused hyphal fragmentation, which may correlate with higher broth viscosity.

**3.9. The Initial Fair Values ( $k_L a$ ) Influence the Physiological Working Capacity Status of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 Mutants.** The initial fair values ( $k_L a$ ) of the AO-M<sub>3</sub>1,

HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants were determined. As shown in Table 4, the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant cells showed rapid growth after inoculation and reached maximal values at 70 h, following the oxygen supply capacity of the cultivation system in all 3 cases. It would be interesting to determine the physiological working capacity status of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants, given that they seem to be only slightly influenced by oxygen supply. The available data show that the maximal biomass-specific productivity of mutants at 150 rpm and 1.10 vvm was slightly higher than at 150 rpm and 0.8 vvm, indicating that the higher initial fair values ( $k_L a$ ) reflect slightly increased biomass accumulation and dextrin consumption.

**3.10. Determination of the Genetic Stability of Mutants.** As shown in Table 5, the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants were evaluated for their genetic stability in terms of AVM and AVM Bla specific productivity (under optimization fermentation parameters). The data show that these mutants could maintain their higher specific productivity after the sixth generation. Moreover, their specific productivity was sustained even as the growth rate increased. The sixth generation of 147-G<sub>5</sub>8 exhibited  $Y_{AVM\text{Bla}}$  and  $Y_{\text{TotalAVMs}}$  values of  $5446 \pm 230$  and  $11089 \pm 460$   $\mu\text{g/mL}$ , respectively. These results suggest that the specific productivity of the strain increased in



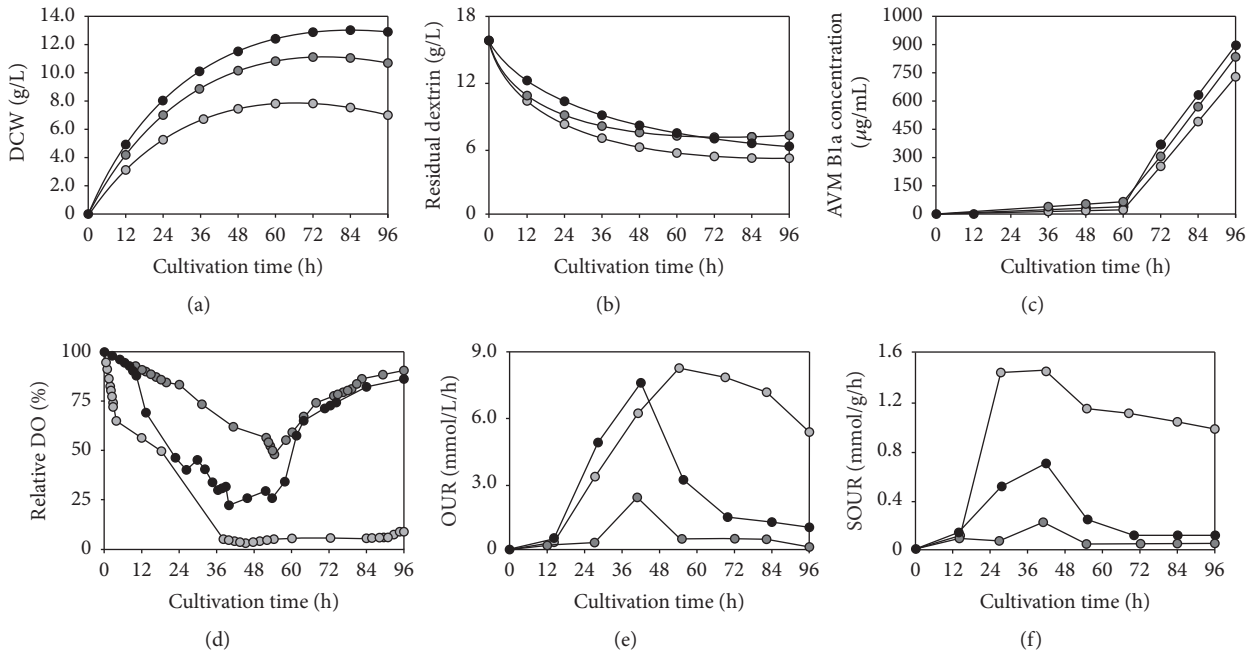


FIGURE 4: Time course of the physiological working capacity of 147-G<sub>5</sub>8 mutants cultivated in stirred-tank bioreactors with different agitation speeds. (a) Time course of cell dry weight with agitation speeds of 150, 250, and 350 rpm. (b) Time course of dextrin consumption with agitation speeds 150, 250, and 350 rpm. (c) Time course of the AVM Bla specific productivity with agitation speeds 150, 250, and 350 rpm. (d) Time course of the dash dotted line of DO-levels with agitation speeds 150, 250, and 350 rpm. (e) Time course of determination of oxygen uptake rate (OUR) with agitation speeds 150, 250, and 350 rpm. (f) Time course of determination of the specific oxygen uptake rate (SOUR) with agitation speeds 150, 250, and 350 rpm. Black circle symbol for 150 rpm speeds, grey circle symbol for 250 rpm speeds, and off-white circle symbol for 350 rpm speeds, respectively.

TABLE 4: The influence of initial fair values ( $k_L a$ ), stir speed, and AVM and AVM<sub>Bla</sub> production on the working capacity of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants under various experimental conditions.

Mutant	Agitation speed (rpm)	Aeration rate (vvm)	Initial fair value $k_L a$ values ( $h^{-1}$ )	Tip speed ( $ms^{-1}$ )	AVM <sub>Bla</sub> production ( $\mu g/mL$ )	Total AVMs production ( $\mu g/mL$ )
AO-M <sub>3</sub> 1	150	1.10	34.4	1.10	3979 ± 110	10366 ± 220
	150	0.80	39.8	1.10	3889 ± 120	9022 ± 120
	250	1.10	42.3	1.20	2254 ± 170	4563 ± 120
	350	1.10	52.6	1.40	1835 ± 120	3843 ± 110
HS-P <sub>1</sub> 8	150	1.10	34.4	1.10	4078 ± 110	9762 ± 180
	150	0.80	39.8	1.10	4232 ± 130	9674 ± 210
	250	1.10	42.3	1.20	2164 ± 110	4713 ± 170
147-G <sub>5</sub> 8	350	1.10	52.6	1.40	1978 ± 100	3689 ± 120
	150	1.10	34.4	1.10	4560 ± 110	10767 ± 230
	150	0.80	39.8	1.10	4770 ± 150	10936 ± 220
	250	1.10	42.3	1.20	2343 ± 130	4892 ± 170
	350	1.10	52.6	1.40	2183 ± 110	4685 ± 160

a dose-dependent manner at 220 AMeV of energy input with 80 Gy dose.

#### 4. Discussion

In recent years, researchers have utilized traditional molecular biological mutation and selection strategies to obtain

improved strains of *S. avermitilis* [36–39]. The new heavy-ion irradiation mutagenesis technique requires input from physics, chemistry, life science, and agronomy [40–42]. However, little data are currently available regarding the effect of heavy-ion irradiation on the production of AVMs and AVM Bla by *S. avermitilis*. Many of the details and effects of this mutagenesis technique were not resolved until now.

TABLE 5: Values of  $Y_{AVM\text{Bla}}$  and  $Y_{Total\text{AVMs}}$  for AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants for different subcultures ( $n = 5$ ).

Mutant	Subcultures	2	3	4	6
AO-M <sub>3</sub> 1	$Y_{AVM\text{Bla}}$ ( $\mu\text{g/mL}$ )	3948 $\pm$ 240	3721 $\pm$ 220	3820 $\pm$ 270	3750 $\pm$ 350
	$Y_{Total\text{AVMs}}$ ( $\mu\text{g/mL}$ )	10297 $\pm$ 290	10090 $\pm$ 330	10459 $\pm$ 210	10730 $\pm$ 280
HS-P <sub>1</sub> 8	$Y_{AVM\text{Bla}}$ ( $\mu\text{g/mL}$ )	4750 $\pm$ 280	4657 $\pm$ 310	4759 $\pm$ 270	4665 $\pm$ 220
	$Y_{Total\text{AVMs}}$ ( $\mu\text{g/mL}$ )	10645 $\pm$ 380	9835 $\pm$ 260	11470 $\pm$ 330	10670 $\pm$ 290
147-G <sub>5</sub> 8	$Y_{AVM\text{Bla}}$ ( $\mu\text{g/mL}$ )	5546 $\pm$ 240	5760 $\pm$ 290	5912 $\pm$ 210	5446 $\pm$ 230
	$Y_{Total\text{AVMs}}$ ( $\mu\text{g/mL}$ )	11320 $\pm$ 380	11982 $\pm$ 350	12067 $\pm$ 310	11089 $\pm$ 460

This scientific report contains a proof-of-concept for the application of different energy levels and doses of  $^{12}\text{C}^{6+}$  heavy ions to generate *S. avermitilis* mutants with high AVM yields to achieve new breakthroughs in the theory and to apply key technology for the breeding and fermentation of these mutants in industrial applications.

The survival rate of *S. avermitilis* is closely related to the mutations that occur after exposure to different energies and doses of  $^{12}\text{C}^{6+}$  heavy ions. Generally speaking, a microbial survivor population of 15% or less may contain mutants. In this study, the original energy and dose of  $^{12}\text{C}^{6+}$  heavy ions were determined, and the resulting survival rates of 13.7%, 8.4%, and 3.4% were compared using a representative set of experimental data. The most prominent feature of most survival curves is a deviation from the dotted line; in other words, the energy- and dose-response curve typically shows shoulder [43–45]. Above an irradiation dose of 80 Gy with energy of 140 AMeV, the surviving fraction does not further increase but rather decreases. As expected, treatment with  $^{12}\text{C}^{6+}$  heavy ions affected survival; an increase in the irradiation energy or dose leads to a decrease in survival, with a final survival value of 3.4% at the strongest tested irradiation. Unexpectedly, as shown in Figures 1(a) and 4(a), we found that the 147-G<sub>5</sub>8 mutants reenter the cell cycle, which is defined as the time period required for cells to grow and divide, rather than completely losing their proliferation capacity [46, 47]. The specific productivity was determined by considering the DCW as the integrated biomass, thus producing integral of the DCW. The growth of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants was comparable. As shown in Figure 1, treatment with 220 AMeV and 80 Gy (147-G<sub>5</sub>8) led to 1.1-fold increase in DCW and 1.7-fold increase in the AVM Bla productivity of these mutants. Tables 1, 2, 3, and 5 show that the mutants within the bulk sample are genetically stable, showing AVMs production levels that increased to approximately 1.6 times that of the original strain. Despite the initial survival rates of 13.7%, 8.4%, and 3.4%, the growth of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant strains was not affected, and their production of AVM Bla and their DCW values both increased considerably.

The HS-P<sub>1</sub>8 and 147-G<sub>5</sub>8 mutants originate from the same parental strain, and their cultivation performance and handling characteristics are similar. We performed a comprehensive study of the protein expression levels over the growth curve, thus making it reasonable to compare how these mutants were affected by treatment with  $^{12}\text{C}^{6+}$  heavy ions. As shown in Figure 3, 2DE and 3DE were used

to identify the most highly expressed soluble proteins in the HS-P<sub>1</sub>8 and 147-G<sub>5</sub>8 mutants. One large and distinct spot was presented in all three mutants, which revealed the existence of a potential highly expressed gene that may be a key gene in AVM and AVM Bla biosynthesis. Due to the DNA damage caused by treatment with  $^{12}\text{C}^{6+}$  heavy ions, DNA repair processes are activated. DNA repair is positively affected by housekeeping genes, such as *aveC*, *aveD*, *aveI*, *atrA*, and *aveR* which are not typically considered to be housekeeping genes; the “housekeeping” term is one typically reserved for genes whose function is needed for the growth and viability of an organism; these (particularly *aveC*, *I*, and *R*) are not essential and are more specifically associated with avermectin production [48–50]. Heavy-ion beam irradiation led to metabolic process upregulating expression of protein, decreasing synthesis inhibition of avermectin Bla, and improving the productivity of avermectin Bla. Unfortunately, there are no detailed studies of these genes. However, many in vitro kinetics studies utilizing cultivation in shake flasks have demonstrated that these housekeeping genes have beneficial effects. For 147-G<sub>5</sub>8, the values for  $Y_{AVM\text{Bla}}$  and  $Y_{Total\text{AVMs}}$  were 5446  $\pm$  190 and 11089  $\pm$  230  $\mu\text{g/mL}$ , respectively. The  $Y_{AVM\text{Bla}}$  and  $Y_{Total\text{AVMs}}$  for 147-G<sub>5</sub>8 and HS-P<sub>1</sub>8 were also significantly higher than those of the original mutant AO-M<sub>3</sub>1.

To further enhance the AVM and AVM Bla specific productivity of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutants and to achieve bioreactor scale-up, the key engineering parameters involved in the operating process were thoroughly evaluated. Three mutants were used in shake flask culture, a simple system that was suitable for substantiating our findings. Parameters such as shaking speed, the OUR, and the SOUR were determined at different filling volumes. As shown in Figures 1, 3, and 4, the AVM Bla specific productivity of the mutants was observed to be much more sensitive to the shaking speed than to filling volume. A shaking speed of only 250 rpm led to normal mycelial growth in the AO-M<sub>3</sub>1 and HS-P<sub>1</sub>8 mutants, while the 147-G<sub>5</sub>8 mutant became much more hairy, with many thicker hyphae. This result demonstrates the dependence on shaking speed and highlights the beneficial effect of an optimal shaking speed on AVM Bla production. Numerous studies have indicated that thicker mycelia may increase oxygen and nutrient transfer within the core zone, thus increasing metabolic efficiency [51–54]. Such an effect may coincide with our observation of the correlation between thicker mycelia and higher AVM Bla production at a shaking speed of 250 rpm. More interestingly,

our results showed that oxygen transfer has a marginal impact on AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 cultivation.

There are several other parameters that affect AVM Bla production in bioreactor scale-up, such as DO, OUR, SOUR, agitation speed, or the initial fair value ( $k_L a$ ). The exact parameters that are used usually depend on the method of measurement and the area of research. How <sup>12</sup>C<sup>6+</sup> heavy-ion treatment at different energies and doses affects production depends on the unknown adaptations of the AO-M<sub>3</sub>1, HS-P<sub>1</sub>8, and 147-G<sub>5</sub>8 mutant strains and their response to cultivation in stirred-tank bioreactors. As discussed above, to further study the effects of various parameters on AVM Bla production in mutant strains, experiments with agitation speeds of 150, 250, and 350 rpm with a fixed aeration rate at 0.8–1.0 vvm were conducted in stirred-tank bioreactors. As shown in Table 4 and Figure 4, the initial fair values ( $k_L a$ ) increased with agitation speed, indicating a positive effect on both OUR and SOUR transfer capacity. Similarly, a higher agitation speed resulted in decreased DCW gain but increased antibiotic production by *Streptomyces clavuligerus*, *Streptomyces hygroscopicus*, *Enterobacter cloacae*, and *Bacillus thuringiensis*. Interestingly, our data showed that AVM Bla production in stirred-tank bioreactors was severely reduced at 350 rpm, reflecting an abnormally high SOUR, while dextrin consumption remained similar to other conditions. Our findings also showed that a higher agitation speed would tear mycelia at the zone near the stirrer and thereby reduce DCW or strongly inhibit secondary metabolism [28, 55, 56]. Our results showed a positive correlation between AVM Bla production and the forms of mycelia permitted by lower agitation intensity. Similarly, the intact pellet morphology had a profound correlation with AVM Bla production; specifically, the fluffy pellets elongated, known as crossover morphology. This morphology was consistent among the mutants and affected the oxygen uptake, the specific oxygen uptake, or the nutrient limitation in the inner area, which may be relieved by these mutants' thicker mycelia or reduced physiologically active pellet size compared to large pellets [57, 58]. Future research should determine whether the mycelial form and prolonged germination time are directly connected during submerged growth in stirred-tank bioreactors.

In conclusion, when using heavy-ion irradiation technology, a new green technology, to generate mutants that produce AVMs and AVM Bla, it is crucial for industrial applications to distinguish between strains with high and low production. This study provides the means to characterize the mutants obtained through <sup>12</sup>C<sup>6+</sup> heavy-ion irradiation and to optimize bioreactor and bioprocess design; it also demonstrates the relationship between the mutants and higher productivity. <sup>12</sup>C<sup>6+</sup> heavy-ion irradiation is a fairly new parameter in process engineering. It was found to affect both mycelial morphology and specific productivity. <sup>12</sup>C<sup>6+</sup> heavy-ion irradiation may provide a reliable and highly active approach to increase the AVM and AVM Bla productivity of *S. avermitilis* in industrial processes. Because of the predictable behavior these mutants showed in response to the energy and dosage of <sup>12</sup>C<sup>6+</sup> heavy-ion irradiation, customized bioreactor and bioprocess designs for

different process appear to be feasible. Because the rheology of the bioprocess and the downstream product processing steps are heavily dependent on strain production ability, carefully determined <sup>12</sup>C<sup>6+</sup> heavy-ion irradiation parameters may prove to be invaluable. The radiation parameters and process data obtained in this study will be helpful for further studies regarding the effective industrial applications and will establish a standard model for such projects or scale-up, thereby facilitating market acceptance and recognition.

## Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Competing Interests

The authors have no conflict of interests to declare.

## Authors' Contributions

Shu-Yang Wang conceived and designed the experiments. Yong-Heng Bo performed the experiments. Shu-Yang Wang, Yong-Heng Bo, and Xiang Zhou analyzed the data. Shu-Yang Wang, Yong-Heng Bo, Ji-Hong Chen, Yu-Chen Wang, Jing Liu, Wei Hu, and Bo-Ling Jiang contributed reagents, materials, and analysis tools to the article. Shu-Yang Wang and Xiang Zhou wrote the paper. Shu-Yang Wang and Xiang Zhou critically revised the manuscript. Shu-Yang Wang, Xiang Zhou, Jian-Ping Liang, Wen-Jian Li, and Guo-Qing Xiao provided the final approval of the version to be published. Shu-Yang Wang, Yong-Heng Bo, and Xiang Zhou contributed equally to this work.

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