Screening of a high-yield strain of avermectin B$_{1a}$ by colony analysis in situ

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Received: 23 January 2022 / Revised: 21 August 2022 / Accepted: 19 September 2022 / Published online: 30 September 2022
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Abstract
Avermectin, an agricultural antibiotic, is widely used as an agricultural insecticide and an important lead compound of antibiotics. It is manufactured by Streptomyces avermitilis through fermentation. Manufacturers pay special attention to screening for strains with high fermentation capacity based on morphological properties of the colony and by the result of shake flask fermentation. These traditional screening methods are time-consuming and labor-intensive and require specialized equipment. Moreover, evaluation of colony appearance is highly subjective. To improve and accelerate the screening process, we developed a rapid in situ screening method. Forty-four strains isolated naturally from the spores of industrial high-yielding strains were studied. The data show that the colony fermentation titer is highly correlated with the yield from the shake flask fermentation of avermectin, and the Pearson’s $R$ is 0.990. The total titer of avermectins by shake flask fermentation is also highly correlated with the B$_{1a}$ titer (Pearson’s $R$ is 0.994). This result also shows that strains can be quickly screened by analyzing the colony titer. Pigment rings of the colonies that appeared after growing and maturing on the new medium plate were analyzed. The chosen colonies were directly marked and punched and then extracted with methanol. The fermentation ability can be evaluated by measuring the absorbance at 245 nm. This methodology can be applied in both natural breeding and mutation breeding conditions. By continuously breeding from 2008 to 2020, the flask titer of avermectin B$_{1a}$ increased from $4582 \pm 483$ to $9197 \pm 1134 \mu g/mL$.

Keywords Avermectin · Abamectin · Screening · In situ · Breeding

Introduction
Avermectin (Fig. 1), produced by Streptomyces avermitilis, is a class of natural compounds with agricultural antibiotic activity with a unique mechanism of action that can effectively control dipteran, homopteran, coleopteran, and lepidopteran pests and a variety of mites (Danaher et al. 2012). Among them, avermectin B$_{1a}$ shows the highest selectivity and safety. At the recommended dose, it is not only safe for humans and livestock but also innocuous to predators and the environment (Bai and Ogbourne 2016; Wang et al. 2019). Therefore, because of its environmental friendliness and low residue, the Ministry of Agriculture of China has retained avermectin while eliminating highly toxic pesticides, with a registered use of avermectin B$_{1a}$ 9–15 g per hectare of field. Recent studies have shown that avermectin also has anticancer, antidiabetic, and antiviral activities and is used in the treatment of several metabolic disorders (El-Saber Batiha et al. 2020, Pfab et al. 2021). In addition, avermectin can be used as a lead compound for the development of pesticides. The commercialized avermectin products are shown in Fig. 1 (Pitterna et al. 2009). The 2015 Nobel Prize in Physiology and Medicine was awarded for the discovery and purification of avermectin and artemisin (Andersson et al. 2015). Currently, the demand for avermectin B$_{1a}$ in the world is at least 5000 tons annually

Key points
• Avermectin is an agricultural insecticide and a lead compound of antibiotics.
• A high-yielding strain can be identified in situ by analyzing the pigment ring around the colony.
• A high-yielding colony can be screened by measuring the methanol extract of the colony at 245 nm.

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For avermectin B$_{1a}$ and B$_{1b}$, the two component chemicals are extremely similar; avermectin B$_{1a}$ has an ethyl group at the C-26 position, while B$_{1b}$ has a methyl group. Because of the similar structure, it is difficult to separate them in the downstream separation process. The commercial standard stipulates that the content of avermectin B$_{1b}$ should be less than 5%, so the ratio of B$_{1a}$/B$_{1b}$ in the fermented product is also an important parameter. A high-yielding strain means lower costs and higher production capacity; consequently, manufacturers are constantly breeding and screening for high-yield strains.

The traditional screening method is to conduct liquid fermentation with shake flask, which is labor-intensive and time-consuming. Moreover, shake flask fermentation requires shaker equipment, which is not suitable for high-throughput screening. Using 96-well plates instead of shake flasks for trial fermentation increases the number of samples that can be screened simultaneously, but it still takes time and effort (Gao et al. 2010a). In comparison to other methods, in situ screening is a faster and easier way to identify a high-yielding strain. This method screens the strain by the morphological characteristics of the colony and the reaction that breaks down the substrate causing a change in appearance in the plate, for example, cellulose-degrading bacteria can be isolated by Congo-Red agar media (Buffo et al. 2021). There are several methods used for in situ screening which are (1) analyzing the size of colonies, (2) detecting the transparent circle around the colonies, and (3) studying the color changes of colonies. The common screening technique is by the presence of the clear halo around the bacterial colonies, which was applied to screen for pectinase-producing strains (Huang et al. 2019; Abdollahzadeh et al. 2020), xylanase strains (Yang et al. 2016), and cellulose-degrading strains. By using this technique, the bacteria which were sensitive to specific enzymes developed the inhibition zone. The inhibition zone is produced in situ and becomes a qualitative method.
to detect antibiotic resistance and industrially to test the ability of solids and textiles to inhibit microbial growth (Bhattacharjee 2015). Spectrum technology has been used in in situ screening. By Raman microscopic and optical tweezers, single cells can be sorted and screened (Wang et al. 2013; Song et al. 2016; Nitta et al. 2020; Yan et al. 2021). The instrument such as a fluorescence-activated cell sorting (FACS) has been used to isolate the required cells (Chen et al. 2013; Nawaz et al. 2015; Mutafopulos et al. 2019) (The isolation chip (or iChip) is a novel in situ method of culturing bacteria (Nichols et al. 2010; Piddock 2015; Sherpa et al. 2015; Lodhi et al. 2018). The soil is diluted in molten agar, and nutrients to isolate a single cell grow in the iChip’s small compartments or wells (Berdy et al. 2017). These techniques simplify the complicated screening workload, save time, and double the screening volume.

Genetic instability is prevalent in Streptomyces avermitilis. It has been reported that 25 of 1300 avermectin-producing strains lost the activity in liquid fermentation (Novak et al. 1993). The manufacturer’s strategy is to screen a large number of strains continuously to improve the quality of the strain and prevent it from degenerating. In fermentation plant, the screening protocol is as follows. The diluted spores are cultured on the plating medium for about 2 days. The colonies with ideal appearance and abundant spores are selected for slant cultivation for another 7–9 days. These slants are inoculated and cultured in shaking seed flasks at 28 °C for about 2 days. The matured seeds are inoculated into fermentation flasks and fermented in shaker for 8–10 days. After fermentation, broth samples are extracted with organic solvent and analyzed by TLC (thin layer chromatography) or HPLC (high-performance liquid chromatography) (Gao et al. 2009; Wang et al. 2017). This screening method is rather subjective, time-consuming, and labor-intensive and requires much equipment. Instead of using flask fermentation, a high-throughput screening technique using 96-well plates was developed, which eliminates a significant proportion of shaking and increases the screening scale but is still time-consuming (Gao et al. 2010a, b). This paper is aimed of shaking and increases the screening scale but is still time-consuming (Gao et al. 2010a, b). This paper is aimed at developing a methodology to screening higher mutation rate compared with the UV and chemical mutagens (Zhang et al. 2014, 2015). In combination with the rapid colony analyses, the culture was continuously bred from one batch to the next batch to obtain a high-yield strain of avermectin B₁₄ₐ.

Materials and methods

Media and culture

The starter strain of Streptomyces avermitilis used in this experiment was provided by Hebei Veyong Biochemicals Co. Ltd. (Gaoceng, Shijiazhuang city, Hebei Province, China). The titer of avermectin B₁₄ₐ in shake flask fermentation was 4582 ± 483 μg/mL. The standard reagent of avermectin B₁₄ₐ with the purity of 99% was also from Hebei Veyong Biochemicals Co. Ltd. ARTP equipment was provided by Tsinghua University (Beijing, China).

The ABK (aspartic acid, beef extract powder, and K₂HPO₄) medium used for isolation contained 1.5% glucose, 0.3% beef extract powder, 0.05% dipotassium hydrogen phosphate, 0.05% aspartic acid, and 2% agar. The PBF (peptide, beef extract paste, and FeSO₄) medium for screening contained 0.56% glucose, 0.2% peptone, 0.1% beef extract paste, 0.1% yeast paste, 0.001% FeSO₄·7H₂O, and 2% agar. The strains on ABK or PBF plates were cultured at 28 °C for 10–12 days. Media used for seeding and fermentation in the flask were prepared according to the literature (Gao et al. 2010a, b). The capacity of the shaking flask used for fermentation was 250 mL, and the liquid volume was 25 mL. The flask fermentation was carried out on a constant temperature shaker (28 °C, 200 rpm) with large eccentricity (the amplitude was 5 mm). The seed culture time was 48 h, and the fermentation duration was 10 days.

Mutation and screening methods

The starter strain was cultured on slant medium (ABK medium, 28 °C) in a test tube for 7 days. The aerial mycelium was scraped and collected by a sterile spatula and washed out with 10 mL of sterile water into a sterile flask. The flask (50 mL) contained 15–25 glass beads (approximately 2–3 mm in diameter), creating a simple ball mill. The collected mycelium was shaken (50 rpm for 5 min every time) and milled in the device for 15 min at least 3 times to completely mill and disperse the spores (the concentration was about 200–1000 spores/mL). One hundred microliters of each dilution was spread on a plate for single colony culture. Mycelium was spread on as many plates as available. The strains with abundant spores and obvious melanin circles were selected for next fermentation.
The strain selected by the above method was transferred and cultured on slant medium of ABK or PBF in a test tube for 7 days. The aerial mycelium was collected by scraping with a sterile blunt spatula, washed out with 15 mL sterile water. The collected mycelium suspension was milled as described above, dispersed and filtered through qualitative filter paper (pore size is about 10 µm). The OD \textsubscript{600} of the filtrated suspension was approximately 0.8 (200 µL filtrate was taken and diluted to 2 mL with ddH\textsubscript{2}O, and was detected by spectrophotometer). High-purity helium (99.99%) was used as the working gas of ARTP. The distance between the source of plasma emission and the substrate spore was 2 mm. The working power was 120 W, and the helium flow rate was 12 L/min. The mycelium was treated for 30 s, 45 s, or 60 s. After mutagenesis, the three groups of mutagenized mycelium solutions were suspended in 20 mL sterile water, shaken with vortex mixer for 1 min, and diluted 10\textsuperscript{1}- to 10\textsuperscript{7}-fold with sterile water. One hundred microliters of sample from each dilution was plated. Multiple samples from each dilution were used for plating. More than 1 mL of the samples from each dilution was used for plating on PBF medium.

**Identification of high-yielding strains by colony morphology**

The treated spores as described above were cultured with sterile loop on ABK medium or PBF medium at 28S \textdegree C for 10 to 12 days. Colonies on the plates were evaluated with a 10\times magnifying glass according to the quantity, size, and appearance of spores. Screening for high-yielding strains was performed daily.

**Rapid screening of colonies**

When the colonies had been cultured on plates for 9 days, they were taken by a puncher and transferred to 5-mL methanol. After ultrasonic extraction for 10 min, the volume was adjusted to 20 mL with methanol. The concentration of the solution was defined as the fermentation titer of the colony. Colonies were numbered and sampled for preservation as they grew. Then, they were obtained with an aseptic puncher and immersed in 5 mL of ethanol. The extraction process was carried out on a shaker at room temperature 150 rpm for 30 min. The supernatant was obtained by filtration through filter paper with 10 µm pore size, transferred to another tube, and the absorbance at 245 nm was detected to evaluate the titer of colony by spectrometer (L6S UV–Vis spectrophotometer, Shanghai Measuretech Instrument Ltd, China). The methanol was taken as the blank control.

**Determination of total AVMs**

Avermectins (AVMs) are a series of compounds produced by the fermentation of Streptomyces avermitilis, including avermectin A\textsubscript{1a}, A\textsubscript{1b}, A\textsubscript{2a}, and A\textsubscript{2b} and avermectin B\textsubscript{1a}, B\textsubscript{1b}, B\textsubscript{2a}, and B\textsubscript{2b}. Ten milliliters of broth from each sample was mixed with 20 mL of methanol, vortexed for 20 min to extract avermectins, and then centrifuged at 3000x g for 5 min. The centrifugation supernatant (5 µL) was applied to TLC analysis with 85% methanol in the mobile phase. The spots of avermectin B and A were scraped out and extracted with methanol, and then the extraction was analyzed with a spectrometer by measuring the absorbance at a wavelength of 245 nm; the avermectin standard sample which is a mixture of avermectin A and avermectin B was provided by Veyong biochemical Co. LTD.

**Determination of avermectin B\textsubscript{1a} titer**

Determination of colony B\textsubscript{1a} titer: About 10 cm\textsuperscript{2} of bacterial lawn was scraped off with a sterile spatula, dispersed and milled in 50 mL of sterile water as previously described with little glass balls. The milled spore suspension was filtered through filter paper. The spore concentration of the filtrate was approximately 10\textsuperscript{8}–10\textsuperscript{9} spores/mL. The collected spores were diluted to about 10–100 spores/mL, and 100 µL was spread on PBF plate medium to culture for 9 days at 28 \textdegree C. The selected colonies were taken by puncher and transferred to 10 mL of methanol. After ultrasonic treatment for 15 min, the volume was adjusted to 20 mL with methanol. The avermectin B\textsubscript{1a} concentration of this sample represents the colony titer and is used to study its correlation with the shake flask fermentation titer.

**Determination of flask fermentation B\textsubscript{1a} titer**

Bacteria seeds were cultured in the seed flask at 28 \textdegree C for 48 h on a shaker, and 2.5 mL was inoculated into the fermentation flask and cultured for 10 days. To extract avermectin, 1 mL of fermentation broth in the shake flask was mixed with 19 mL of 95% (v/v) ethanol. Vortexing and ultrasonication were performed 3 times for 5 min each. The mixture was centrifuged at 3000 g/min for 15 min. The supernatant was filtered through a nylon syringe filter (0.25 µm Ø Unichro product, Hangzhou, China) and analyzed by HPLC (Agilent 1100 with diode array detector). The detection wavelength was 245 nm, and the analytical column was a Shim-Pack CLC-ODS column (150×6.0 mm 5 µm). The mobile phase consisted of methanol (42.5%), acetonitrile (42.5%), and water (15%). The flow rate was 1.0 mL/min, injection volume was 1 µL, and the data was analyzed with Agilent ChemStation. B\textsubscript{1a}/B\textsubscript{1b} value is obtained by calculating the peak areas of B\textsubscript{1a} and B\textsubscript{1b} in HPLC spectrum.
Results and discussion

Screening in situ by pigment ring in PBF plate

The spores of the original strain were diluted and plated for culturing. Colonies were selected for shake flask fermentation. We found that the fermentation ability of offspring was highly variable.

As shown in Fig. 2, strains grown on PBF medium plates produced black pigments around the colony (Fig. 2a), which was absent when cultured on ABK medium plates (Fig. 2b). Therefore, colonies grown on ABK medium were selected based on the colony size and spore quantity. The diameter of mature colony is generally greater than 4 mm, the normal spore color is gray or white, and the bare colonies are degenerate colonies. In addition to these characteristics, the colonies on the PBF medium plates could be selected based on the size and depth of the pigmentation ring. In the avermectin fermentation plant, strain engineers focus on studying and summarizing the relationship between fermentation ability and colony morphology, including growth state, color, and spore quantity (Ping-Ping et al. 2017). Generally, there are three types of colonies of *Streptomyces avermitilis*, namely, the gray powder type, white type, and bald type. Among them, the strain with gray powder type colonies usually has the highest production capacity. In contrast, the strain lacking visible mycelium was grown to bald colony and usually was poor in fermentation. On PBF medium plates, 44 strains were selected according to the spore quantity and the black pigmentation ring. Among them, 16 strains produced 3000–4000 μg/mL avermectin B1a, and only two strains fermented more than 4000 μg/mL B1a (Fig. 3), which confirms the genetic instability of *Streptomyces avermitilis* described in the literature (Xi et al. 2020). Without an efficient screening method, the trait of avermectin production will degenerate quickly with passaging.

The correlation between the colony titer on the plate and the shake flask fermentation unit was studied, and the results are shown in Fig. 4a. The regression equation is \( Y = 3.705X + 122.63 \) (\( Y \) stands for the shake flask fermentation titer of B1a, \( X \) stands for the colony fermentation titer of B1a on the plate), and the Pearson \( r \) value is 0.990, indicating a positive correlation. The correlation between the titer of total avermectin and the titer of avermectin B1a was analyzed (Fig. 4b), which shows that AVMs = 11.9 + 2.51B1a (Pearson \( r = 0.994 \)). This high correlation means that high-yield strains of avermectin B1a can be identified simply by measuring the total titer of avermectin on the plate.

It has been proven that there is a negative correlation between the production ability of secondary pigments and...
avermectin. The *ave*I gene positively regulates the production of pigments, and deletion of the *ave*I gene led to increased biosynthesis of avermectin B1a by approximately 16-fold (Chen et al. 2008). Another study indicated that *ave*I functioned as a global regulator in *S. avermitilis* and controlled not only secondary metabolism and morphological differentiation but also primary metabolism (Chen et al. 2009). Consequently, on PBF medium plates, colonies with abundant spores, plump morphology, and smaller pigmentation rings tend to be high-yielding colonies. However, in the application, the pigment circle is closely related to the growth of the colony (Fig. 2). The larger the colony grows, the larger the pigment circle, and the smaller the colony, the smaller the pigment circle. Therefore, it was necessary for us to further develop a relatively objective in situ screening method.

Avermectin B1a is usually detected by HPLC, whose pretreatment is tedious, and the process is rather complex, labor-intensive, time-consuming, and not suitable for high-throughput screening. Fortunately, the titer of the colony on the plate shows a very close correlation with the titer of the shake flask culture, and so does the B1a titer and the total titer of AVMs. Therefore, we can develop an in situ screening method by analyzing the titer of the colony on the plate.

**Rapid screening of colonies**

Avermectins are intracellular products that mainly exist within the mycelium of *Streptomyces avermitilis*, and very little avermectins are present in the supernatant of the fermentation. However, during fermentation, many brown pigments accumulate in the culture, which have strong UV absorption at 200–300 nm wavelengths. We were concerned that water-soluble substances such as these pigments might be co-extracted by methanol and potentially interfere with the rapid analysis of colonies. Therefore, we analyzed the UV absorption of these water-soluble impurity substances. Fifty milliliters of avermectin fermentation broth was centrifuged at 3000 × g for 5 min, and the supernatant was analyzed by UV scanning. We found that the maximum absorption wavelength of the impurity was 254 nm (Fig. 5). The maximum absorption wavelength of avermectin B1a is approximately 245 nm. The punctured colony with agar was extracted with methanol, and the supernatant of the extraction was also scanned. The data in Fig. 6 show that most absorption at 200–300 nm wavelengths. We were concerned that water-soluble substances such as these pigments might be co-extracted by methanol and potentially interfere with the rapid analysis of colonies. Therefore, we analyzed the UV absorption of these water-soluble impurity substances. Fifty milliliters of avermectin fermentation broth was centrifuged at 3000 × g for 5 min, and the supernatant was analyzed by UV scanning. We found that the maximum absorption wavelength of the impurity was 254 nm (Fig. 5). The maximum absorption wavelength of avermectin B1a is approximately 245 nm. The punctured colony with agar was extracted with methanol, and the supernatant of the extraction was also scanned. The data in Fig. 6 show that most
of the chromatograms had a maximum absorption peak at approximately 245 nm. Two samples lost the ability to produce both pigment and avermectin (Fig. 6). The ideal strain should produce only avermectin \( \text{B}_{1a} \) without producing any pigment at all because these pigments make industrial wastewater hard to treat (Hong et al. 2011). In this study, we did not find such an ideal strain in our mutant library. This maximum absorption peak at 245 nm can be used to determine the avermectin content, which can be used to screen for high-yielding strains in situ.

The data of in situ colony screening are shown in Fig. 7, and the colony from the original strain was chosen as a control. The absorbance of the control group was 0.33 ± 0.04. Those above the control level were considered the desirable and positive mutants. The majority of mutants were negative and undesirable, and only approximately 6.7% of strains carried the positive mutation. Sample No. 18 showed the highest absorption, reaching more than 0.5, a 51.5% increase compared to the control. This strain was selected and cultured for further study. DNA is a double-stranded structure. Mutations often cause base changes in only one strand, leaving the other strand intact (Lieber 2010). As a result, there will be at least two genotypes in the same colony. We therefore further diluted the spores of the high-yield strains obtained from the preliminary screening and discarded the heterozygotes to obtain pure high-yield strains. All of the screened strains were studied by flask fermentation, and the results are shown in Fig. 8. The flask titer of avermectin \( \text{B}_{1a} \) of the start strain was only 4582 \( \mu \text{g/mL} \), and that of AVMs was 10,587 \( \mu \text{g/mL} \). By screening the library of ARTP-induced mutants, we selected a strain with a flask titer of avermectin \( \text{B}_{1a} \) of 6137 \( \mu \text{g/mL} \) and AVMs of 16,919 \( \mu \text{g/mL} \). By secondary screening, the average flask titer of avermectin \( \text{B}_{1a} \) increased to 7017 \( \mu \text{g/mL} \), and AVMs increased to 17,027 \( \mu \text{g/mL} \). The flask titers of avermectin \( \text{B}_{1a} \) and AVMs were maintained during the next two generations of fermentation, suggesting stable inheritance of this excellent trait in the mutant.

In situ screening technology is widely used in microbiology. It is well known that \textit{Penicillium spp.} was discovered by observing the plate in situ (Diggins 1999). Bacteriostatic circles and transparent circles can be classified as in situ screening techniques. In fact, in situ screening is often the first choice for screening a special strain because it is fast, intuitive, and scalable. However, strains of avermectin are notoriously hard to screen in situ, and there was no report of successes prior to this study. We considered two effective methods: one is to examine the pigmentation ring on the PBF medium plate, and the other is to analyze the colony extract. QPix™ Microbial Colony Picker can be applied directly on PBF medium plates. High-throughput analysis of colony extracts can be performed in 96-well plates, combined with background subtraction of 254 nm absorption, to achieve fast and accurate assessment of strain performance.

For industrial manufacturing, an excellent avermectin strain should not only produce more avermectin \( \text{B}_{1a} \) but also have a high \( \text{B}_{1a}/\text{B}_{1b} \) ratio. In the separation process, avermectin \( \text{B}_{1a} \) is purified by the crystallization process. Because of the similarity between avermectin \( \text{B}_{1a} \) and avermectin \( \text{B}_{1b} \) in chemical structure and solubility, if the concentration of \( \text{B}_{1b} \) is too high, the content of \( \text{B}_{1b} \) in the product is also high, and \( \text{B}_{1b} \) is an undesirable impurity in the product. Thirty from the separated strains were selected for flask fermentation analysis (Fig. 9). Although we did not deliberately screen for high \( \text{B}_{1a}/\text{B}_{1b} \) strains, the majority of strains isolated naturally had a higher \( \text{B}_{1a}/\text{B}_{1b} \) ratio than the control group. This may be due to the high \( \text{B}_{1a}/\text{B}_{1b} \) ratio of the starting strain. As a result, despite \( \text{B}_{1b} \) also having a peak absorption at 245 nm, high \( \text{B}_{1a} \) strains can still be screened out by this method.
Finally, the inconsistency of colony performance in shake flask fermentation versus in industrial large-scale fermentation should be discussed. In laboratory settings, strains were selected by colony potency analysis and shake flask fermentation tests. However, their high yield and other favorable characteristics are often not reproduced when cultured in large fermenters in industry. We have experienced this phenomenon in our previous work with an avermectin manufacturer. We sent several excellent strains to the plant for trial fermentation. These strains in general are better than the strains they were using, but it is interesting to note that the best performer in the fermenter was often not the strain with the highest performance in the lab; instead, the second highest-producing lab strain was the best in the fermenter (unpublished data). Shaking flasks and fermenters are very different in many aspects. In addition to temperature and nutrient supply, oxygen availability is vital to the growth of microbes and cells in culture. In shaking flasks, the transfer of oxygen takes place via two liquid surfaces, and the efficiency is determined by the size and shape of the flask, agitation speed, filling volume, and ambient conditions. Cultivation processes in shaking flasks are never under absolute control, as they are not monitored in real time for critical parameters such as temperature, dissolved oxygen, or optical density. Temperature and dissolved oxygen, for example, can only be regulated by controlling ambient conditions in a sealed shaker system or a cultivation room. Automated processes, such as feeding the culture according to defined profiles or the integration of control loops, cannot be accomplished. The fermenter is equipped with multiple sensors that allow for real-time monitoring. Temperature, dissolved oxygen, pH, and biomass are measured constantly and displayed numerically and graphically. Exhaust, metabolites, and redox potential can be calculated based on these measured parameters in the fermenter. Various types of impellers operating at specific agitation speeds can meet the individual requirements of different cell types regarding shearing force and mixing efficiency (Hitesh, Karteek et al. 2010). Strains sensitive to shearing force may produce poorly in fermenters but better in flasks. Antifoaming agents are necessary in fermenters to reduce the surface viscosity of the film, increase the velocity of drainage, and enhance the diffusion of gas. However, sensitive strains tend to age easily in the presence of defoamers and strong shearing force, which are
likely the causes of loss of genetic stability, persistent poor fermentation, and suboptimal performance in fermenters. Therefore, it is necessary to add a certain amount of defoamers, such as soybean oil and silicon polyether, to the media during screening to help select strains with good tolerance to shearing force and defoamers. This could be tested in future studies to improve our screening method.

In support of the significance of shearing force, our lead authors have made the following observation while working for an avermectin manufacturer (unpublished work, Hebei Veyong Biochemical Co., LTD). In 2000, the titer of avermectin was only approximately 1800 μg/mL, and the fermentation process could not continue for a long time because of hyphal aging. When the shaft of a fermenter broke by accident, stirring had to be stopped. The whole fermentation process was carried out only by blowing sterile air. While this fermentation unit was expected to be particularly low due to this accident, we were pleasantly surprised that the fermentation titer of avermectin was drastically improved in subsequent analysis. The hyphae in this particular fermentation broth appeared globular under the microscope because high-speed stirring is not conducive to mycelium ball formation. This suggests that Streptomyces avermitilis is sensitive to shearing force, causing the discrepancy between the titer in a flask culture and in a fermenter. This also implies that the airlift fermenter may be better for Streptomyces avermitilis fermentation. There have been some previous studies on the effects of stirring on fermentation (Zhang et al. 2010). The correlation between the colony titer and the shake flask titer was studied in detail by Cheng (Cheng, Liu et al. 2020). Eleven single colonies were fermented in shaking flasks, and their potency was analyzed by HPLC. The correlation coefficient was 0.924 between the titer of a single colony and the titer of the shaking flask. At the significance level of 0.05, there was a positive correlation between the single colony titer and the corresponding shake flask titer. Despite the difference in medium compositions, moisture contents, and modes of nutrient transfer, plates and flasks are nonetheless highly correlated in terms of fermentation level.

Conclusions

Streptomyces avermitilis is genetically unstable and easily degenerates. Active strain screening is very important to maintain its fermentation ability. The colony fermentation titer was highly correlated with the shake flask fermentation of avermectin, and the Pearson R was 0.990. The total titer of avermectins from shake flask fermentation was also highly correlated with the B1a titer (Pearson R is 0.994). By analyzing the UV scanning spectrum of the fermentation broth and the spectrum of the methanol extract of the colony, the optimal wavelength for screening was identified. By analyzing the ultraviolet absorption of the colony methanol extract at 245 nm, the strains can be quickly screened in situ.

The spores of Streptomyces avermitilis were treated with ARTP to establish a mutant library. Avermectin was extracted from the colony with methanol, and the absorbance was measured at 245 nm. A strain with 51.5% higher absorbance was selected. The fermentation ability of avermectin B1a of this strain was as high as 7017 μg/mL, which was an increase of approximately 55.1% compared to that of the original strain.

Acknowledgements The authors are very grateful for the helpful discussion provided by Jianhui Gao and Xi Cheng from Hebei Xingbai Pharmaceutical Group Co. Ltd.

Author contribution GZX conceived and designed the research and wrote the manuscript. LJH, HF, and ZB conducted the experiments. LZX and XTY contributed analytical tools.

Funding This study was funded and financially supported by the Funds of Huai’an Municipal Science and Technology Bureau (grant number HAN201610).

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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