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# Effects of *Magnaporthe oryzae* cell-free filtrate on the secondary metabolism of *Streptomyces bikiniensis* HD-087: a non-targeted metabolomics analysis

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**Abstract**

Rice blast, a disease caused by *Magnaporthe oryzae*, significantly threatens global rice production. To improve the anti-*M. oryzae* activity of *Streptomyces bikiniensis* HD-087 metabolites, the effects of inducer, *Magnaporthe oryzae* acellular filtrate, on secondary metabolism of *S. bikiniensis* HD-087 were studied. The results showed that *M. oryzae* cell-free filtrate cultured for 96 h served as the most effective inducer, significantly enhancing the anti-*M. oryzae* activity of metabolites of *S. bikiniensis* HD-087 and increasing the diameter of the inhibitory zone by 2.96 mm. The inhibition rates of *M. oryzae* colony diameter and spore germination in the induced group were 12.39% and 39.6% higher than those in the non-induced group, respectively. Metabolomic profiling of strain HD-087 highlighted substantial differences between the induced and non-induced groups. At 48 h of fermentation, a total of 705 distinct metabolites were identified, while at 96 h this number decreased to 321. Moreover, induction markedly altered primary pathways such as the tricarboxylic acid cycle, amino acid biosynthesis, and fatty acid metabolism in *S. bikiniensis* HD-087. qPCR analysis showed that *nrps* genes and *pks* genes in the induced group were significantly up-regulated by $9.92 \pm 0.51$ and $2.71 \pm 0.17$ times, respectively, and biotin carboxylase activity was also increased 26.63%. These results provide a theoretical basis for using inducers to enhance the antimicrobial ability of *Streptomyces*.

**Keywords** *Streptomyces*, *Magnaporthe oryzae*, Induced culture, Secondary metabolites, Metabolomics

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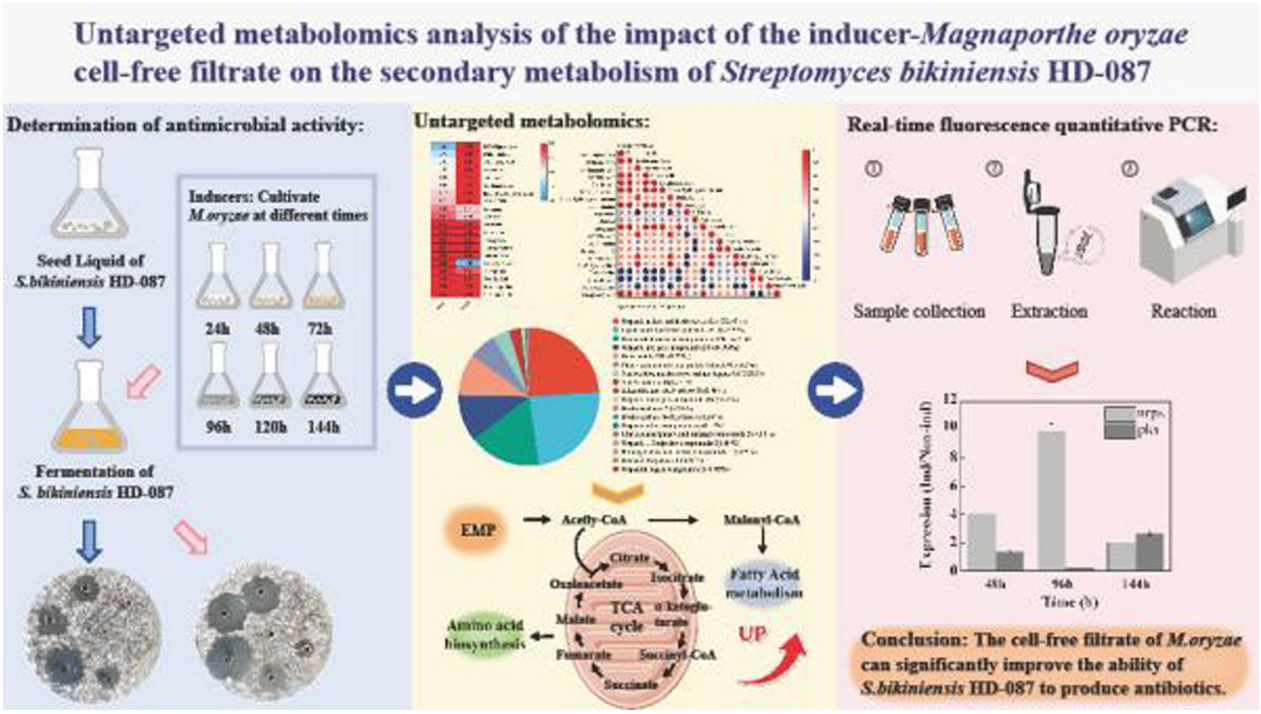
**Graphical Abstract**




## Introduction

Rice blast, caused by the fungal pathogen *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*), is a disease that can devastate rice production, significantly impacting both yield and grain quality [2]. Beyond rice, *M. oryzae* exhibits a broad host range, infecting other economically important crops such as wheat and barley [13]. The global significance of this pathogen was highlighted in 2012 when the international molecular plant pathology community ranked it among the top ten fungal pathogens threatening global food security [11]. Thus, effective management of *M. oryzae* is crucial for safeguarding food security worldwide [12].

Among the microorganisms employed for plant disease control, the genus *Streptomyces* is highly regarded for its ability to synthesize diverse and efficacious secondary metabolites [25]. These metabolites include antibiotics, enzymes, polysaccharides, organic acids, siderophores, and other bioactive compounds, making *Streptomyces* an important source of biocontrol agents [30, 39] and plant growth promoters [21]. Although these substances are extensively studied and utilized, *Streptomyces* metabolites specifically developed for rice production remain scarce. Kasugamycin has been a successful example in controlling rice blast over the past few decades [31]. Its long-term application has not resulted in significant bioaccumulation within organisms or to the widespread emergence of resistant pathogens. However, prolonged use may heighten the risk of resistance development among pathogenic fungi through co-evolutionary dynamics. Consequently, there is an urgent need to develop novel agricultural antibiotics targeting rice blast. Enhancing pharmaceutical diversification could serve as an effective strategy to mitigate future disease outbreaks and associated risks and achieve sustainable disease control [15, 38, 43].

However, the discovery of novel antibiotics from conventional *Streptomycetes* is increasingly difficult due to the high rediscovery rates stemming from extensive screening efforts, coupled with limitations inherent in traditional methodologies. Advances in modern genomics suggest that certain genes remain unexpressed or exhibit low expression under standard culture conditions [48], referred to as "silent genes" that can be activated under specific circumstances [37, 49]. Bode et al. [5] proposed the "One Strain–Many Compounds" (OSMAC) strategy, which emphasized the manipulation of culture parameters, including nutrient composition and environmental factors as well as co-culturing techniques and epigenetic

modulation, to fully exploit microbial biosynthetic potential. Among these approaches, co-culturing is particularly notable as an effective method of activating cryptic biosynthetic pathways through interspecies interactions like physicochemical communication and competition, leading to novel secondary metabolite production [7, 36]. The co-culture approach offers a relatively straightforward means of enhancing bioactive compounds' yields while unlocking chemical diversity, without the need for complex genetic manipulation or costly reagents [33]. Nevertheless, pure culture processes are undoubtedly simpler than co-culture methods. The direct utilization of microbial-produced inducers may therefore more effectively stimulate the expression of silent or latent genes within producers themselves.

Our previous research demonstrated that antimicrobial compound production by *S. bikiniensis* HD-087 could be significantly enhanced using *M. oryzae* and its metabolites as inducers, the latter exhibiting superior efficacy [26, 27]. To further identify effective inducers, the present study evaluated how various time-point supernatants from *M. oryzae* cultures influence metabolite profiles within *S. bikiniensis* HD-087 cultures via comprehensive metabolomic analyses comparing induced (Ind) versus non-induced (Non-ind) states, providing a theoretical basis for inducer-based development of new targeting antibiotics against rice blast.

## Materials and methods

### Test strains and culture media

*Streptomyces bikiniensis* HD-087 was isolated from soil samples collected from Hulunbeier Grassland, Inner Mongolia. It's draft genome sequence accession number in GenBank is PRJNA823498. *M. oryzae* was kindly donated by Dr Chong Zhang from Shenyang Agricultural University. Gauze's synthetic broth medium No. 1 (soluble starch 20 g/L, $KNO_3$1 g/L, $K_2HPO_4$ 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, NaCl 0.5·g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, pH 7.0–7.2) [41] was used for the activation of *S. bikiniensis* HD-087. DBY medium (glucose 20 g/L, soybean flour 5 g/L, yeast flour 4 g/L, ammonium sulfate 5 g/L, NaCl 1g/L, and $K_2HPO_4$ 0.05 g/L) [14] was used to obtain the fermentation products of *S. bikiniensis* HD-087. PDB [10] and CM [44] media were used for the liquid culture of *M. oryzae.*

### Preparation of inducers

The cell-free filtrate of *M. oryzae* was used as an inducer to stimulate the secondary metabolism of *S. bikiniensis* HD-087. The culture supernatant of *M. oryzae* was prepared according to the method of [26, 27]. One milliliter of spore suspension of *M. oryzae* with a concentration of $1\times10^5$ spores/mL was inoculated into 50 mL of PDB medium and incubated in a shaker at 180 r/min for 144 h. During the incubation period, 2 mL of the spores was sampled at 24 h intervals, and the samples were centrifuged at 10,000 r/min for 10 min and filtered (filter pore size: 0.45 μm) to remove the mycelium. According to the different culture time, the supernatants of *M. oryzae* cultured for 24, 48, 72, 96, 120, and 144 h were named as inducers Mo-24, Mo-48, Mo-96, and so on.

### Preparation of *S. bikiniensis* HD-087 metabolites

The fermentation of *S. bikiniensis* HD-087 in DBY medium was divided into two groups: one for the Ind culture and the other for the Non-ind culture (control). In the Ind group, 1-mL of the inducer, which was cultured at different times, was taken and added to the corresponding triangular flasks containing 50 mL of DBY medium, respectively. While the Non-ind group was added the same amount of PDB medium. Then, 2 mL of *S. bikiniensis* HD-087 seed solution was inoculated simultaneously in all treatments. Preparation of seed solution: one full ring of fresh slanting spores of strain HD-087 was inoculated into 50 mL Gauze's synthetic broth medium No. 1, cultured at 180 r/min and 28 °C for 36 h.

Both Ind and Non-ind groups were incubated in a shaker at 28 °C for 144 h at 180 r/min, and samples were taken at 24-h intervals from 48 h onwards. The samples were centrifuged at 10,000 r/min for 20 min, the precipitate was discarded, and the supernatant was filtered to remove mycelium. The final supernatant obtained was the metabolite to be measured.

### Analysis of the induction effect of inducers

The effects of different inducers on the antimicrobial activity of *S. bikiniensis* HD-087 metabolites were analyzed using three methods.

Oxford cup diffusion: A 3-mL aliquot of *M. oryzae* spore suspension ($1\times10^5$ mL) was added to 50 mL of CM medium at a temperature of 50 ℃, and mixed thoroughly. The mixture was then poured into sterile 15-cm Petri dishes and allowed to solidify. Sterile Oxford cups were placed on the solidified agar, and 200 μL of each test sample was added to the cups. Uninoculated DBY medium served as a negative control. The plates were incubated at 28 °C for 72 h, and the diameters of the inhibition zones were measured. Inhibition zone diameter was used as an indicator of antimicrobial activity. Each treatment was repeated 3 times.

Mycelial growth inhibition assay: 1 mL of *S. bikiniensis* HD-087 culture supernatant, obtained from Ind and Non-ind cultures after 96 h of fermentation, was added to sterile Petri dishes (9-cm diameter) containing 25 mL of CM medium cooled to 50 °C, respectively. The plates were gently swirled to ensure homogenous mixing and

allowed to solidify. To eliminate excess moisture, the plates were inverted and incubated overnight. Then, using a sterilized puncher, 6-mm diameter fungus blocks were extracted from the edge of a *M. oryzae* colony that had been incubated normally on CM medium at 28 ℃ for 10 days, and inoculated into the center of the previously mentioned plates containing different HD-087 supernatants. At the same time, a CM agar plate without HD-087 supernatants was inoculated as a control. All plates were incubated at 28 °C for 10 days, and colony morphology was assessed. The *M. oryzae* colony diameter (CD) of each plate was measured using the cross method. Each treatment was repeated 3 times.

Spore germination inhibition assay: The 50-μL quantities of *M. oryzae* conidial suspension (approximately 10–20 spores per field of vision) were added to sterilized concave slides. The supernatants of Non-ind and Ind groups were then added to respective concave slides, mixed well with the conidial suspensions, cultured at 28 °C for 3 h and 3h 30 min (3.5 h), and then sampled and observed with a microscope according to Lau and Hamer [24]. Each treatment was replicated three times, and 200 spores were examined randomly in each treatment (germination was considered to have occurred when the germ tube was larger than the short radius of the spores).

### LC–MS/MS untargeted metabolomics analysis

#### *Preparation of samples*

(1)(1)(1)(1) Preparation of samples for metabolomics analysis of *M. oryza*e inducers.

The samples of *M. oryzae* inducers cultured for 48 h, 96 h, and 144 h were prepared according to Sect. "Preparation of inducers": 100 μL of each sample was aspirated into a 1.5-mL centrifuge tube, and then 400 μL of extraction solution (acetonitrile:methanol = 1:1) containing 0.02 mg/mL of the internal standard (L-2-chlorophenylalanine) was added.

The samples were mixed by vortex for 30 s, low-temperature sonicated for 30 min (5 °C, 40 kHz), then placed at − 20 ℃ for 30 min. Next, the samples were centrifuged for 15 min (4 ℃, 12,000 r/min), and the supernatant was removed and blown dry under nitrogen. The sample was then re-solubilized with 100 μL of solution (acetonitrile:water = 1:1) and extracted by low-temperature ultrasonication for 5 min (5 °C, 40 kHz), followed by centrifugation at 12,000 r/min and 4 °C for 10 min. The supernatant was transferred to sample vials for LC–MS/MS analysis.

(2)(2)(2)(2) Sample preparation for metabolomics analysis of intracellular metabolites of *S. bikiniensis* HD-087

The intracellular metabolites of *S. bikiniensis* HD-087 were treated in two groups: the Ind culture and the Non-ind culture. Two fermentation time nodes, 48 h and 96 h, were set for each treatment. In this way, a total of four groups of metabolites of *S. bikiniensis* HD-087 were harvested. According to the receiving time and group were named Non-ind 48, Non-ind 96, Ind 48 and Ind 96 cultures.

First, the fermentation broth of *S. bikiniensis* HD-087 was obtained according to Sect. "Preparation of S. bikiniensis HD-087 metabolites", centrifuged at 9,500 r/min for 10 min to remove the supernatant, washed three times with PBS, centrifuged to obtain the precipitate, and dispensed into new 1.5-mL centrifuge tubes. Then 50 mg of each sample was added to a 2-mL centrifuge tube and a 6-mm diameter grinding bead was added. Next, 400 μL of extraction solution (methanol:water = 4:1 (v:v)) containing 0.02 mg/mL of internal standard (L-2-chlorophenylalanine) was used for metabolite extraction. Samples were ground in the Wonbio-96c (Shanghai Wonbio Biotechnology Co., LTD) frozen tissue grinder for 6 min (− 10 °C, 50 Hz), followed by low-temperature ultrasonic extraction for 30 min (5 °C, 40 kHz). The samples were left at − 20 °C for 30 min, centrifuged for 15 min (4 °C, 13,000 g), and the supernatant was transferred to the injection vial for LC–MS/MS analysis.

#### *LC–MS/MS analysis*

The samples were analyzed using LC–MS/MS on a Thermo UHPLC-Q Exactive HF-X system (Shanghai Meiji Biomedical Technology Co., Ltd.) with the following parameters: (1) Chromatographic conditions: 3 μL of the sample was separated on a HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, USA) and then entered into mass spectrometry; the mobile phases consisted of 0.1% formic acid in water:acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5, v/v) (solvent B), the flow rate was 0.40 mL/min, the column temperature was 40 ℃, and the injection volume was 3 μL; (2) mass spectrometry conditions: the mass spectrometric data were collected using the Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode and negative mode, with a mass scan range of 70–1050 m/z. The optimal conditions were set as follows: aux gas heating temperature of 425 ℃; capillary temperature of 325 ℃; sheath gas flow rate of 50 psi; aux gas flow rate of 13 psi; ion-spray voltage floating (ISVF) of − 3500 V in negative mode and 3500 V in positive mode,

respectively; normalized collision energy; 20–40-60 eV rolling for MS/MS. The full MS resolution was 60,000, and the MS/MS resolution was 7,500. Data acquisition was performed in Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 70–1050 m/z.

#### *Identification and analysis of metabolites*

The UHPLC-MS raw data were converted into the common format using Progenesis QI software (Waters, Milford, USA) through baseline filtering, peak identification, peak integral, retention time correction, and peak alignment. The data matrix containing sample names, m/z, retention time, and peak intensities was exported for further analyses. At the same time, database searches were conducted to identify the metabolites, the main databases being the HMDB (http://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/) and the self-compiled Majorbio Database (MJDB) of Majorbio Biotechnology Co., Ltd. (Shanghai, China). The metabolites with VIP > 1, $p < 0.05$ were determined to be significantly different based on the variable importance in the project (VIP) obtained by the OPLS-DA model and the p-value generated by Student's t test.

### Analysis of *S. bikiniensis* HD-087 *nrps* and *pks* gene expression through qPCR

The total RNA of *S. bikiniensis* HD-087 was extracted from the induced and non-induced cultures separately using Trizol (Beyotime Biotechnology Co., LTD., Shanghai, China). cDNA templates were synthesized by reverse transcription using a kit (Vazyme Biotechnology Co., LTD., Nanjing, China). Primers were designed based on the sequences of the key secondary-metabolism genes of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) in the whole genome of *S. bikiniensis* HD-087, using 16S rRNA as the reference gene. The primers were synthesized by Sangon Biotechnology Co. LTD. (Shanghai, China). Real-time fluorescence quantitative PCR (qPCR) was performed with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster, CA, USA), using SYBR green as a fluorescent marker. The qPCR procedure was as follows: pre-denaturation at 95.0 ℃ for 30 s; denaturation at 95.0 ℃ for 30 s, annealing at 57.0 ℃ for 20 s, and extension at 72 ℃ for 15 s.

### Method for determination of biotin carboxylase

Enzyme activity was assessed using an enzyme-linked immunosorbent assay detection kit. For the analysis, the supernatant of the fermentation broth from the Non-ind group and the Ind group cultured for 24, 48, 72, 96, 120, and 144 h were extracted for measurement. Please refer to the instruction manual of the reagent kit for specific operating methods.

### Data processing and analysis

All experiments were repeated 3 times. Data are expressed as mean ± standard deviation. Multiple comparisons of ANOVA test data were performed using JMP (Version 10.0.10) software. The significance levels for statistical tests were set at $< 0.01$ and $< 0.05$ to indicate highly significant and significant differences, respectively, and asterisks were used to indicate significant differences between samples. Origin 2022 software was used for statistical and graphical analyses.

## Results and analyses

### Inducers significantly increased the anti-*M. oryzae* activity of *S. bikiniensis* HD-087 metabolites

The results of the effect of inducers on the antimicrobial activity of *S. bikiniensis* HD-087 metabolites are shown in Fig. 1. The 48-h metabolites from the Non-ind group did not show inhibitory activity against *M. oryzae* (Fig. 1A). In contrast, the 48-h metabolites in the Ind group, including all of the inducers from Mo-24 to Mo-l44, showed significant resistance to *M. oryzae*, all with inhibition diameters greater than 20 mm. The results suggested that all inducers could advance the initial antibiotic production time of *S. bikiniensis* HD-087 by 48 h. The inhibition diameter of Ind (Mo-96) was the largest among all treatments at any fermentation time, indicating that the inducer Mo-96 (the supernatant of *M. oryzae* cultured for 96 h) had the best induction effect on antibiotic production of *S. bikiniensis* HD-087. The antifungal diameter of Non-ind and Ind (Mo-96) was compared longitudinally during their respective fermentation processes, and it was found that the diameters of both peaked at 96 h (Fig. 1B). However, it is worth noting that the inhibitory zone of Ind (Mo-96) was 2.96 mm larger than that of Non-ind at this time (Fig. 1B). Although all inducers stimulated the early (48-h) expression of antibiotics, their effects showed clear individual differences. With the exception of the inducer Mo-96, the antibiotic production of the other inducers was not satisfactory at 72 h to 96 h of fermentation. The different effects of inducers on the antibiotic synthesis of *S. bikiniensis* HD-087 indicated that their chemical composition is significantly different.

The CD results showed that the supernatants of *S. bikiniensis* HD-087 in Ind (Mo-96) inhibited *M. oryzae* colony expansion more strongly than those in Non-ind. When the CD of the CK reached 70.27 ± 1.23 mm

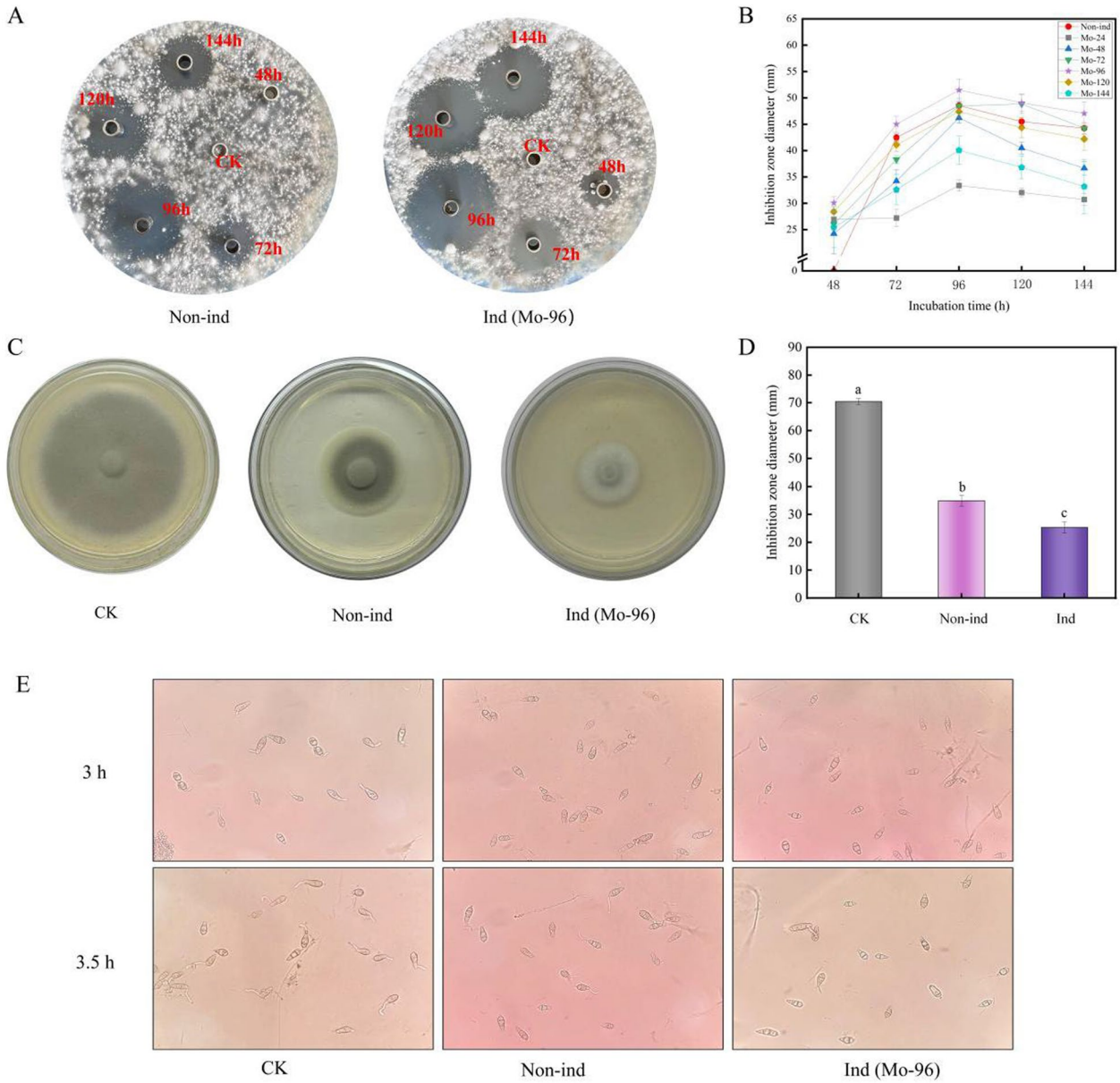



**Fig. 1** Comparison of anti-*M. oryzae* effects of *S. bikiniensis* HD-087 supernatant obtained under ind and non-ind conditions. **A** The Anti-*M. oryzae* zone of different treatment: Ind (Mo-96) represents the group was induced with the supernatant of *M. oryzae* cultured for 96 h; **B** Anti-*M. oryzae* results line chart, Non-ind represents the group treated with non-inducers; Mo-24 ~ Mo-144 represent those groups induced with different inducers involving the supernatant of *M. oryzae* cultured for 24 ~ 144 h, respectively; **C** Inhibitory effect of Ind group and Non-ind group on colony expansion of *M. oryzae*; **D** Column chart of inhibitory effect on colony expansion; **E** The effect of induction culture on the germination of *M. oryzae* spore

(Fig. 1C), the CD of the Ind and Non-ind groups were 25.97 ± 0.56 mm and 34.68 ± 0.74 mm, respectively. The inhibition effect on *M. oryzae* CD in the Ind group was 12.39% higher than that in the Non-ind group. In addition, the colony color indicated that Ind supernatant could significantly inhibit the formation of melanin in *M. oryzae*, while Non-ind supernatant did not have this effect.

The spore germination assay (Fig. 1E, Table 1) showed that the inhibiton rate of *M. oryzae* spore germination at 3 h of Non-ind group reached 71.01%, and that of Ind group reached 86.54%. At 3h 30 min (3.5 h) of cultivation, the inhibition rate of spore germination rate of Non-ind reached 53.13%, while that of Ind reached 74.17%. The inhibition rate of spore germination in Ind group was

**Table 1** The effect of induction culture on spore germination of *M. oryzae*

| Incubation time | Treatment | Spore germination rate (%) | Inhibiton rate of spore germination (%) |
|---|---|---|---|
| 3 h | CK | 73.20 ± 3.05a | – |
| | Non-ind | 22.12 ± 5.16b | 71.01 |
| | Ind | 9.85 ± 1.67c | 86.54 |
| 3 h 30 min(3.5 h) | CK | 81.42 ± 3.14a | – |
| | Non-ind | 38.16 ± 0.69b | 53.13 |
| | Ind | 21.03 ± 2.39c | 74.17 |

39.6% higher than that in Non-ind group at 3h 30 min (3.5 h).

### Analysis of PCA and PLS-DA of *M. oryzae* inducers and *S. bikiniensis* HD-087 metabolites

Principal component analysis (PCA) score of the *M. oryzae* inducers based on the first two principal components (PC) reached 68.81% (PC1 59.30%, PC2 9.51%) in positive (POS) mode (Fig. 2Aa) and 67.70% (PC1 56.50%, PC2 11.20%) in negative (NEG) mode (Fig. 2Ab). Notably, all biological replicates fell within the 95% confidence circle of the sample. This indicated a high level of similarity within the group and significant differences between the groups, resulting in a cumulative difference ($R^2X(cum)$) of 0.763 and 0.772 for POS and NEG, respectively, with intergroup differences. Moreover, the PLS-DA model exhibited strong goodness of fit ($R^2Y(cum)$) (POS 0.990, NEG 0.993) and high predictability ($Q^2(cum)$) (POS 0.963, NEG 0.520) (Fig. 2Ac, d). A cross-test was performed on the PLS-DA model (Fig. 2Ae, f)., and the intercept of the $Q^2$ fitted line on the y-axis was less than 0 (POS − 0.102, NEG − 0.221), indicating that the model did not undergo an overfitting condition. The replacement test passed.

The *S. bikiniensis* HD-087 metabolite group had a PCA score of 48.70% (PC1 33.70%, PC2 15.00%) in POS mode and 53.90% (PC1 32.80%, PC2 21.10%) in NEG mode (Fig. 2Ba). All biological replicates fell within the 95% confidence circle of the sample, indicating a high level of similarity within the group and significant differences

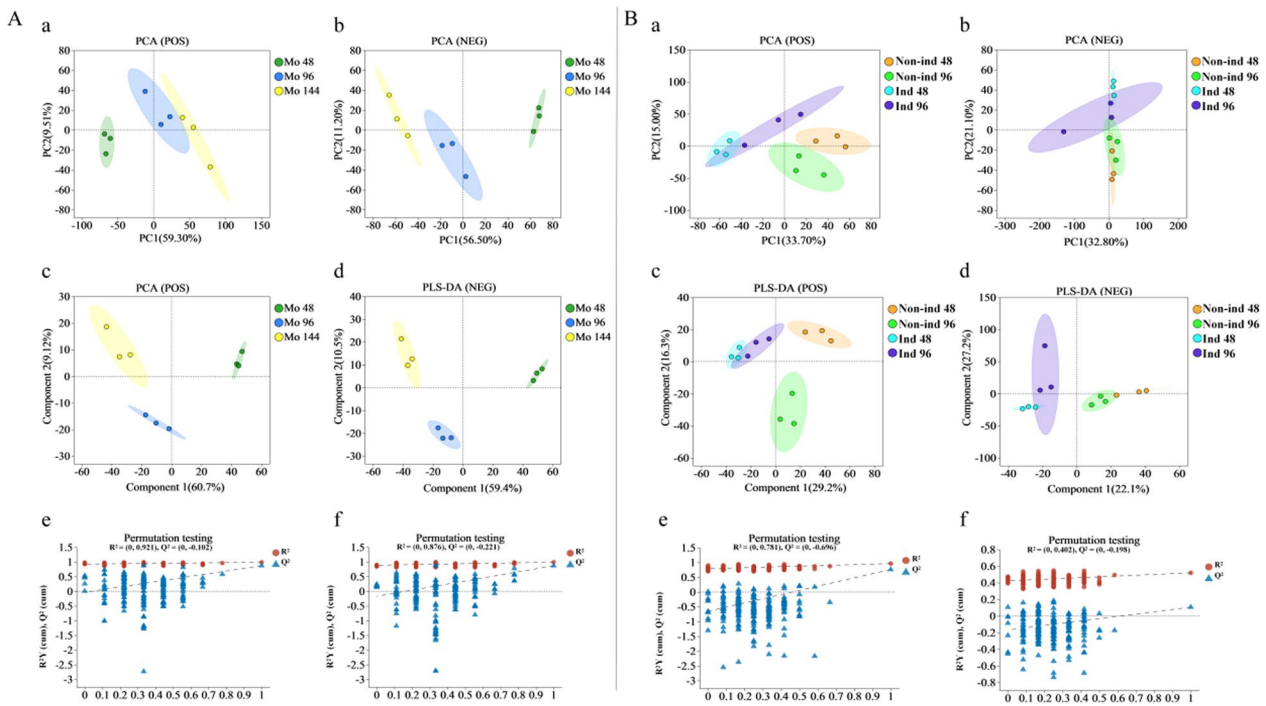



**Fig. 2** PCA and PLS-DA analysis of *M.oryzae* inducers and *S. bikiniensis* HD-087 metabolites. **A** (**a**, **b**) PCA score plots of *M. oryzae* in POS (**a**) and NEG mode (**b**); (**c**, **d**) Validation of PLS-DA models of pairwise comparation among *M.oryzae* in POS (**c**), and NEG mode (**d**); (**e**, **f**) the x-axis represents the retention of the permutation test, while the y-axis represents the values of the permutation test. **B** (**a**, **b**) PCA score plots of *S. bikiniensis* HD-087 in POS (**a**) and NEG mode (**b**); (**c**, **d**) Validation of PLS-DA models of pairwise comparation among *M.oryzae* in POS (**c**), andNEG mode (**d**); (**e**, **f**) the x-axis represents the retention of the permutation test, while the y-axis represents the values of the permutation test. R2Y (cum) represents the cumulative explained variance in the model for the Y matrix, and $Q^2$ (cum) represents the predictive ability of the model. The closer these values are to 1, the more reliable the model. The two dashed lines represent the regression lines for the explained variance and predicted values of the Y matrix. $R^2$ and $Q^2$ represent the correlation coefficient of the regression line and the intercept value of the y-axis, respectively

between the groups (Fig. 2Bb). The cumulative difference in $R^2X$(cum) in POS and NEG modes was 0.624 and 0.657, indicating a between-group difference. The results of the PLS-DA model exhibited strong goodness of fit ($R^2Y$(cum)) (POS 0.873, NEG 0.871) and high predictability ($Q^2$(cum)) (POS 0.763, NEG 0.107) (Fig. 2Bc, d). A cross-test was performed on the PLS-DA model (Fig. 2Be, f), and the intercept of the $Q^2$ fitted line on the y-axis was less than 0 (POS − 0.696, NEG − 0.657), indicating that the model did not suffer from an overfitting condition. The replacement test passed.

### Inducers differed significantly between incubation times

The compound compositions of the inducer Mo-96 were significantly different to those of Mo-48 and Mo-144. Of these compounds, the top 20 compounds that could serve as signaling molecules were screened out based on their substantial differences (Fig. 3A). Among them, 15 compounds in Mo-96 demonstrated up-regulation relative to both Mo-48 and Mo-144, with 9 compounds showing up-regulation greater than 1.6 times: phosphoric acid, norepinephrine, abscisic acid, epinephrine, salicylic acid, prostaglandin 12, sphinganine, aspartic acid, and adenosine. These 9 substances, primarily hormones and acids, play crucial roles in cellular processes by acting as signaling molecules that initiate cascade reactions regulating gene expression. They also influence the intracellular environment through modulation of acid–base balance and energy homeostasis, both of which are essential for optimal cellular function. Consequently, these molecules, particularly phosphoric acid and norepinephrine, which exhibited the most pronounced up-regulation, warrant further investigation as potential key triggers of antibiotic production in *S. bikiniensis* HD-087.

A correlation analysis (Fig. 3B) was conducted on the expression levels of these 20 signaling molecules at 96 h ($P < 0.001$ indicating highly significant correlation), revealing a strong correlation among a diverse array of substances. For instance, there was a notably significant correlation between phosphoric acid, the most significantly up-regulated substance, and 7 other signaling molecules, including adenosine, beta-alanine, and oxoglutaric acid; a highly significant correlation between norepinephrine and dopamine was also observed. These findings suggest that such molecules may synergistically regulate antibiotic production in *Streptomyces*.

### Metabolites significantly differed between Ind and Non-ind cultures of *S. bikiniensis* HD-087

Metabolomics analysis, utilizing the HMDB 4.0 database for annotation, identified a total of 2,148 metabolites across both Non-ind and Ind groups of *S. bikiniensis* HD-087 (Fig. 4A). These metabolites were categorized into 18 distinct groups, with organic acids and their derivatives representing the most abundant category, at 23.93%, followed closely by lipids and lipid-like molecules, at 23.79%. Veen diagrams, constructed to visualize the overlap of metabolites under varying culture conditions and fermentation time points (Fig. 4B), illustrated significant alterations in the *S. bikiniensis* HD-087 metabolome induced by *M. oryzae* cell-free filtrate, suggesting that these metabolic shifts likely

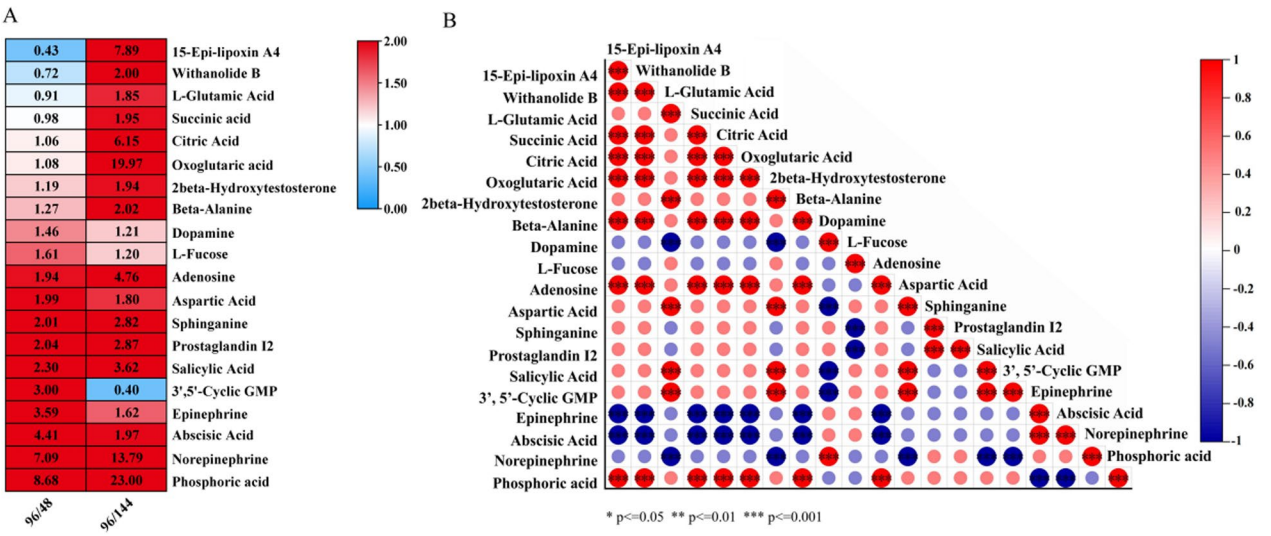



**Fig. 3** Heat maps and correlation analysis of differential compounds in *M. oryzae* inducers. **A** Expression calorigram of differential signal molecules; **B** Correlation analysis between differential signal molecules. Red indicates high abundance levels, while blue indicates lower abundance level

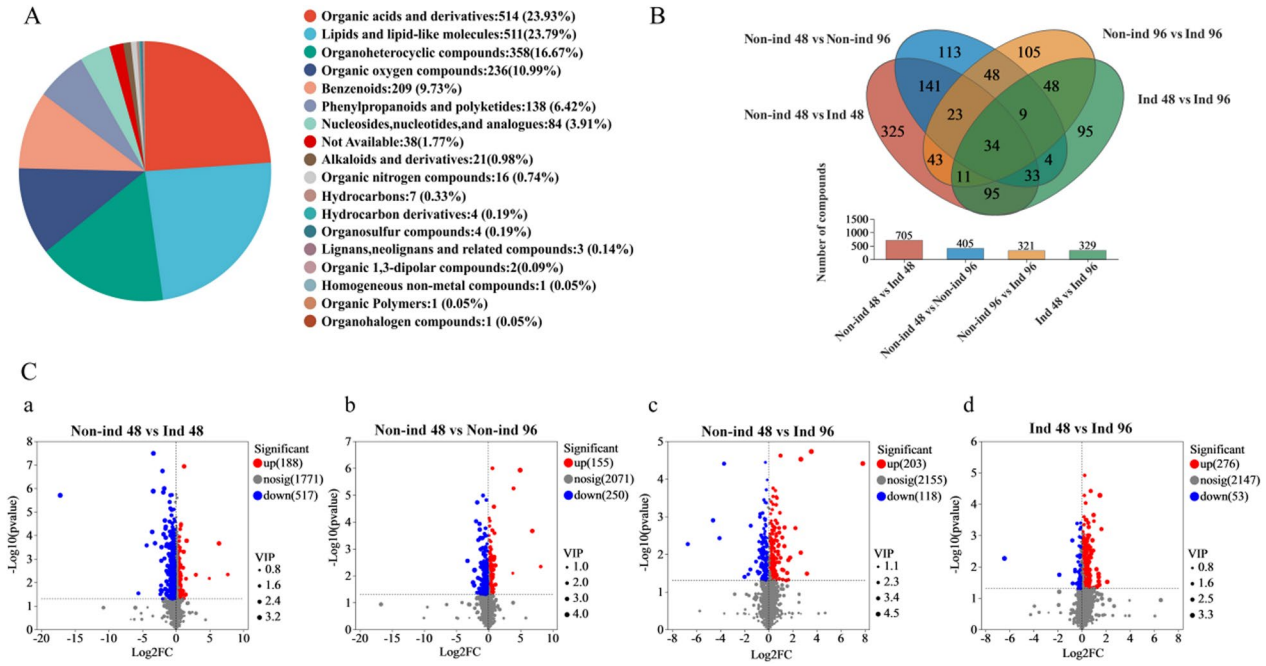



**Fig. 4** Differential metabolite analysis. **A** HMDB compound classification statistics of metabolites; **B** Veen diagrams of significant differential metabolites; **C** Volcano plots of significant differential metabolites between ind non-ind groups in different times

contributed to the observed enhancement in antifungal activity. The volcano plot (Fig. 4C) indicated that inducers significantly influenced metabolite production in *S. bikiniensis* HD-087. A total of 705 differential metabolites were identified between the Ind and Non-ind groups at 48 h of fermentation; among these, 188 were up-regulated while 517 were down-regulated (VIP > 1 and $P < 0.05$). The up-regulated compounds may serve as critical evidence for the early synthesis of antibiotics within Ind. After extending fermentation to 96 h, a total of 321 differential metabolites remained between the Ind and Non-ind groups, 203 of which were up-regulated and 118 down-regulated. This suggested that the up-regulated substances may have been responsible for the enhanced antimicrobial activity observed in the Ind group compared to its Non-ind counterpart.

Moreover, notable changes in metabolite profiles occurred at different fermentation durations within each group: Specifically, there were a total of 405 different metabolites detected between samples taken at 48 h and 96 h in the Non-ind group, out of which 155 exhibited up-regulation while 250 showed down-regulation. In contrast, the Ind group displayed 329 different metabolites over 48 h versus 96 h of fermentation, with 276 being up-regulated and 153 down-regulated. The increased metabolites observed at 96 h are posited as primary contributors to superior bacteriostatic efficacy noted during this extended fermentation.

### Cluster analysis of *S. bikiniensis* HD-087 differential metabolite hierarchies

Furthermore, we performed metabolite hierarchical cluster analysis on the top 50 significantly different metabolites based on expression abundance (Fig. 5). It was divided into 5 subclusters (subclusters 1–5). The analysis revealed a significant increase in the relative abundance of subclusters 1, 3, and 4 in the Ind group compared to the Non-ind group. Conversely, the relative abundance of subclusters 2 and 5 was decreased in the Ind group. Within the three subclusters (1, 3, and 4) that exhibited increased relative abundance, a total of 17 metabolites were identified as organic acids and their derivatives, predominantly comprising amino acids, peptides, and related compounds. Additionally, 8 potential substances were classified as lipids or lipid-like entities, while 2 potential substances fell under nucleosides and nucleotides. These substances are intricately linked to the growth and metabolic processes of the organism.

### KEGG pathway enrichment of *S. bikiniensis* HD-087 differential metabolites

To elucidate the potential biosynthetic pathways associated with differential metabolites in *S. bikiniensis* HD-087, the differential metabolites produced under Non-ind and Ind conditions at different times were annotated and analyzed using the KEGG database,

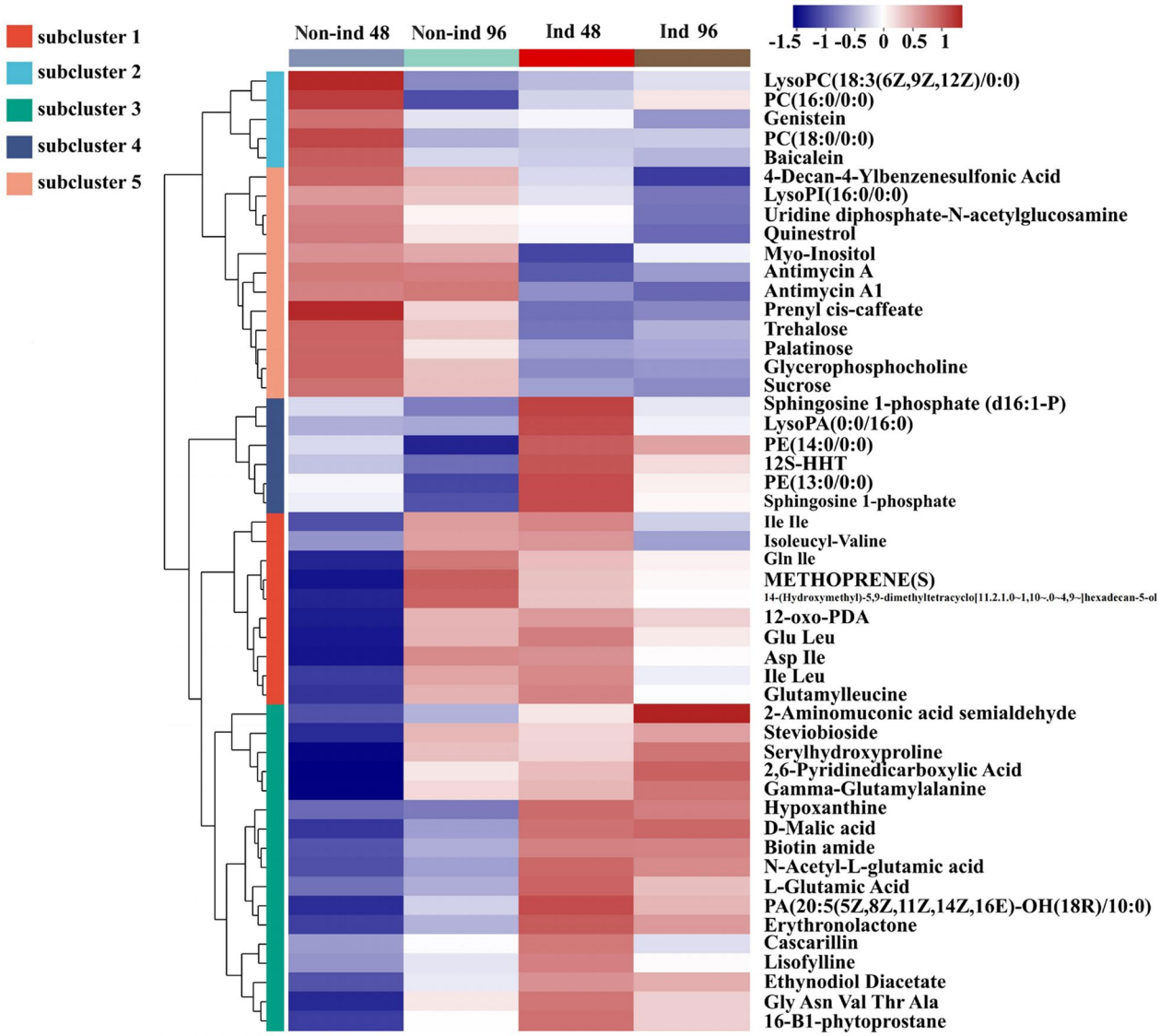



**Fig. 5** Hierarchical clustering analysis significant differential metabolites. (Top 50 based on metabolic abundance.) Each row in the figure represents a potential metabolite, each column represents a sample, and the color represents the relative abundance of potential metabolites within the group. Red indicates high abundance levels, while blue indicates lower abundance level

resulting in their classification into 20 KEGG secondary pathways (Fig. 6A). Of these, 816 potential metabolites from 11 secondary pathways were classified as "metabolites." In metabolism, the top three pathways in terms of proportion were "Amino acid metabolism," "Biosynthesis of other secondary metabolites," and "Chemical structure transformation maps." The 11 secondary pathways are closely related to antibiotic synthesis. Among them, "Amino acid metabolism," "Lipid metabolism," "Carbohydrate metabolism," "Metabolism of cofactors and vitamins," "Metabolism of other amino acids," "Nucleotide metabolism," "Xenobiotics biodegradation and metabolism," and "Energy metabolism" can provide important precursors, cofactors, and energy for antibiotic synthesis. Notably, "Biosynthesis of other secondary metabolites" and "Metabolism of terpenoids and polyketides" are direct pathways of antibiotic synthesis. This suggests that *M. oryzae* inducers induce/stimulate antibiotic synthesis in *S. bikiniensis* HD-087 by influencing a series of pathways associated with antibiotic biosynthesis.

In addition, metabolic pathway enrichment was performed on the different substances fermented at different times between the Ind group and the Non-ind group.

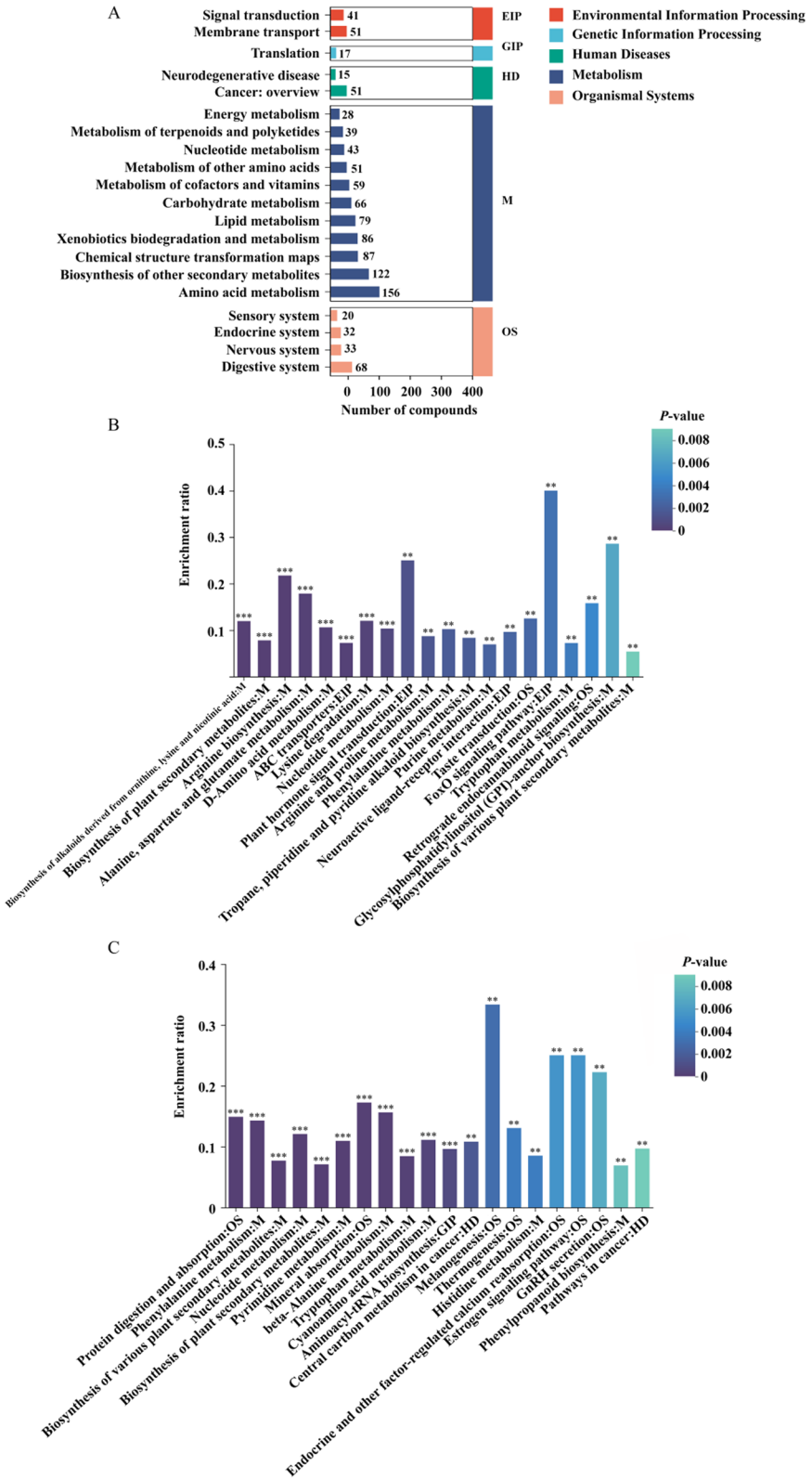



**Fig. 6** KEGG analysis of significantly different metabolites. **A** KEGG pathway annotated and classification; **B** KEGG enrichment analysis at 48 h of Ind and Non-ind groups; **C** KEGG enrichment analysis at 96 h of Ind and Non-ind groups

The results showed that at 48 h of fermentation, differences in metabolite enrichment between the two groups were observed in the "Biosynthesis of plant secondary metabolites," "ABC transporters," and "D-Amino acid metabolism" pathways (Fig. 6B). At 96 h of fermentation, the enriched metabolites that differed between the two groups belonged to the "Biosynthesis of various plant secondary metabolites" and "Tryptophan metabolism" pathways (Fig. 6C) ($P < 0.001$). These findings demonstrated that the *M. oryzae* inducer significantly affected secondary metabolism in *S. bikiniensis* HD-087, potentially leading to enhanced antibiotic production.

### Inducers increased the abundance of antimicrobial and potentially antimicrobial substances in *S. bikiniensis* HD-087

A total of 33 substances with reported antimicrobial activities were screened from the metabolites that differed between the Ind and Non-ind groups (Fig. 7A). Among these, a total of 16 metabolites were up-regulated at 96 h, the top three of which were encecalin, aucubin, and carbazole. In addition, 25 metabolites with increased abundance and potential antimicrobial activity were screened out in the Ind group (Fig. 7B), the top three of which were coniferyl acetate, melanostatin, and apigenin 7-sulfate. This further demonstrated that the inducers significantly affected the synthesis of secondary antimicrobial compounds in *S. bikiniensis* HD-087. In particular, coniferyl acetate in the Ind group was 209 times higher than in the Non-ind group, It is known that coniferyl alcohol has specific antifungal effects, but there are few studies on coniferyl acetate. We speculated that coniferyl acetate also has significant antifungal activity, and we would focus on its antifungal effects in future studies.

### Inducers affected the abundance of *S. bikiniensis* HD-087 metabolites associated with metabolic pathways upstream of antibiotic synthesis

Since the synthesis of secondary metabolites is closely related to upstream primary metabolites (Fig. 8A), we analyzed the effects of inducers on metabolites related to the tricarboxylic acid (TCA) cycle, amino acid biosynthesis, and fatty acid metabolism pathways, and found that the abundance of these substances changed in response to the inducers (Fig. 8B). In the fatty acid metabolic pathway, there was a significant difference in the expression of three fatty acids, with hexadecanoic acid being down-regulated at both 48 h and 96 h while tetradecanoic and octanoic acid were up-regulated. Five differential metabolites were found in the TCA cycle, namely acetyl-CoA, citrate, isocitrate, α-ketoglutarate,

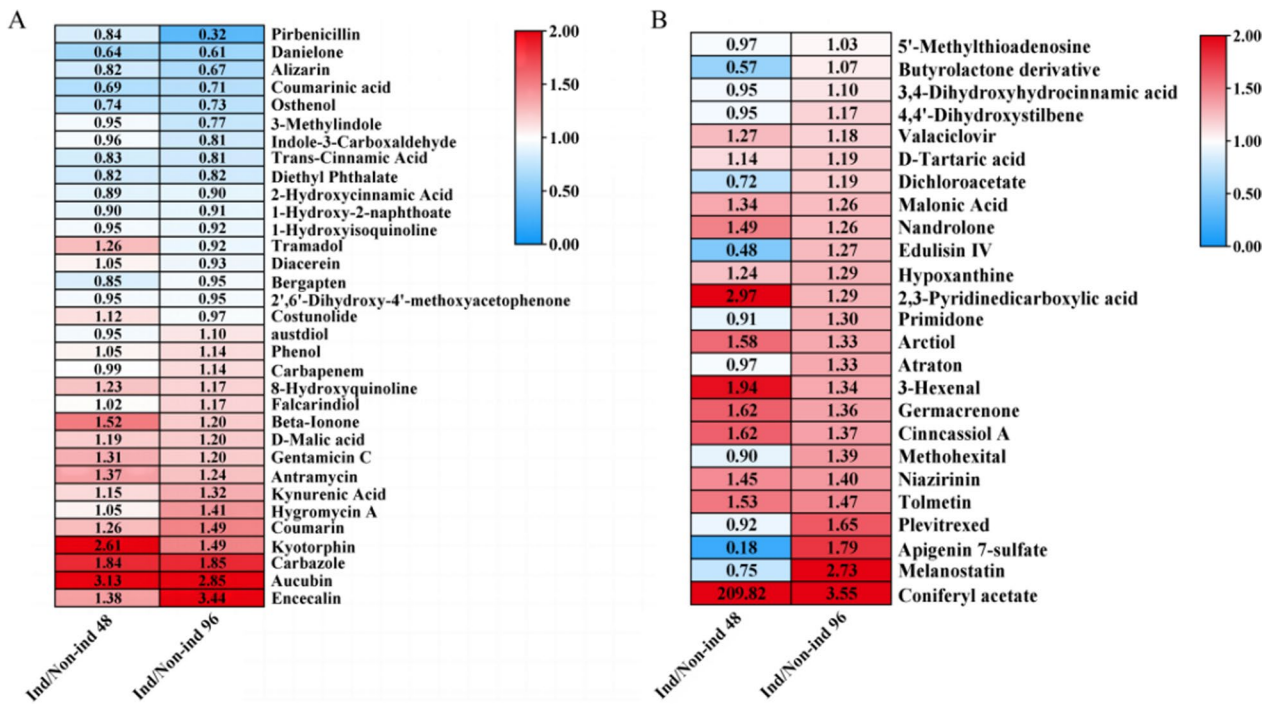



**Fig. 7** Abundance heat maps of known and potential antimicrobial substances in the Ind and Non-ind groups. **A** Substances with antimicrobial activity have been clearly reported; **B** Substances that have not been clearly reported but may have antimicrobial activity

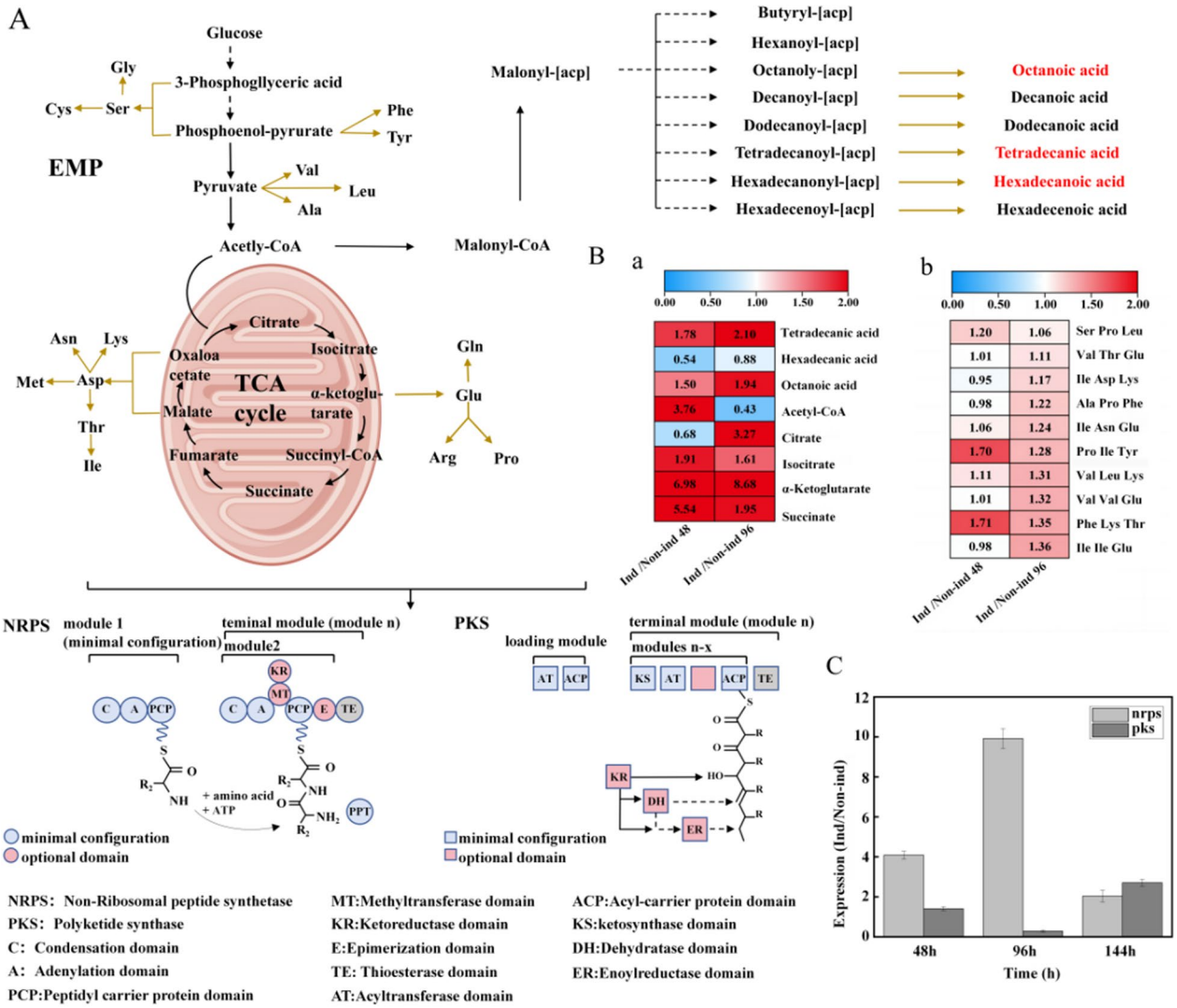



**Fig. 8** The effect of induction culture on the synthesis of secondary metabolites. **A** NRPS and PKS use primary metabolites to synthesize secondary metabolites; **B** expression of differential metabolites of the EMP pathway, pyruvate metabolism, TCA cycle, and fatty acids; Expression level of amino acids; **C** Expression level of amino acids; **D** qPCR results of *nrps* and *pks*

and succinate. Acetyl-CoA, a central metabolite involved in various metabolic pathways, plays a crucial role in maintaining physiological homeostasis. In the Ind group (Mo-96), acetyl-CoA levels were elevated at 48 h but decreased at 96 h compared to the Non-ind group, which partly explained the change in secondary metabolites in the Ind group. In the amino acid biosynthesis pathway, the up-regulation of two amino acid groups—"Phe, Lys, Thr" and "Pro, Ile, Tyr"—was remarkable, suggesting that the inducers may have exerted an important influence on the biosynthesis of secondary metabolites of *S. bikiniensis* HD-087 by regulating the synthesis of these two groups, which often serve as precursor molecules in the synthesis of peptides and lipopeptides.

### Effect of inducers on *nrps* and *pks* gene expression in *S. bikiniensis* HD-087 detected by qPCR

Since NRPS and PKS are able to synthesize lipopeptide and polyketide antibiotics from primary metabolites (Fig. 8C), we examined the effects of inducers on *nrps*(code NRPS) and *pks*(code PKS) gene expression in *S. bikiniensis* HD-087. The qPCR analysis revealed the inducer Mo-96 significantly up-regulated *nrps* gene expression in *S. bikiniensis* HD-087 (Fig. 8C), and the up-regulation amplitude was the highest ($9.92 \pm 0.51$ times) at 96 h of fermentation. This result can be corroborated with the results in Sect. "Inducers significantly increased the anti-M. oryzae activity of S. bikiniensis HD-087 metabolites" (the antifungal zone of

the supernatant fermented for 96 h in the Ind (Mo-96) subgroup was not only the largest in the fermentation process, but also significantly larger than that of the Non-ind group). In contrast, the expression of *pks* gene showed a wave effect, up-regulated at 48 and 144 h and down-regulated at 96 h. Notably, the *M. oryzae* inducers had a stronger activation effect on *nrps* compared to *pks*, suggesting a preferential influence on the biosynthesis of peptides and lipopeptide antibiotics (regulated by *nrps*) over polyketides (regulated by *pks*). This observation aligned with previous findings reported by [26, 27].

#### *Determination of biotin carboxylase activity*

Biotin carboxylase plays a crucial role in various biological processes such as fatty acid metabolism, amino acid metabolism, and carbohydrate metabolism, and is one of the key enzymes that maintain normal metabolic activity in organisms. Therefore, it also plays an indispensable regulatory role in the synthesis of downstream antibiotics. With the extension of cultivation time, the activity of biotin carboxylase showed a trend of first increasing and then decreasing (Fig. 9). The maximum enzyme activity of the Ind group appeared at 48 h, while that of the Non-ind group appeared at 72 h. And the maximum enzyme activity value of the Ind group was significantly higher than that of the Non-ind group by 26.63%. It suggested that *M. oryzae* inducers can affect the production of secondary metabolites such as antibiotics by influencing the primary metabolism of *S. bikiniensis* HD-087.

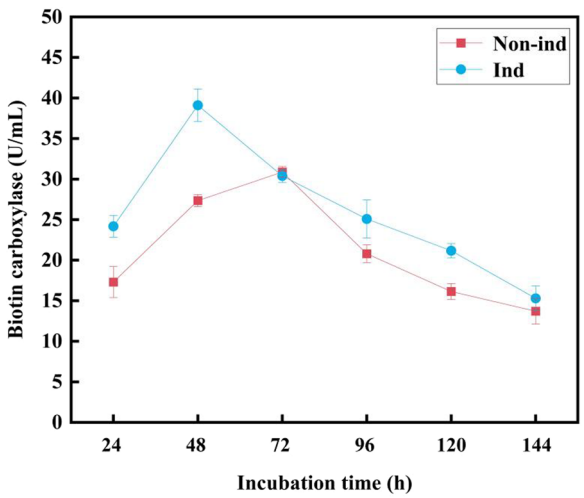



**Fig. 9** Determination of biotin carboxylase activity

## Discussion

A strain of *Streptomyces* usually contains 30–40 biosynthetic gene clusters of secondary metabolites, but these genes are often silent and their expression is regulated by endogenous and environmental factors. Activating the expression of silenced genes and increasing their expression levels can generate new antibiotics and improve their yield. Using inducers to enhance the expression of antibiotic synthesis genes is simple, cost-effective, easy to promote, and has good application prospects. This has been confirmed through several experiments, for example, the marine *Streptomyces cinnabarinus* PK209 can produce antifouling diterpene.lobocompactol, and Cho et al. added a small volume of 16-h-old *Alteromonas* sp. KNS-16 culture to the 96-h-old PK209 culture caused rapid induction of lobocompactol production, 10.4-fold higher than that collected from a single PK209 culture [9]. Onaka et al. [34] found that mycolic-acid-containing (MAC) bacteria could activate the silent gene cluster of *Streptomyces* and promote the synthesis of natural metabolites. Among these MAC bacteria, *Tsukamurella pulmonis* TP-B0596, *Rhodococcus erythropolis*, and *Corynebacterium glutamicum* all affected the biosynthesis of *Streptomyces* to varying degrees, with *Tsukamurella pulmonis* TP-B0596 inducing the production of a new antibiotic, alchivemycin A, by *Streptomyces endus* S-522. However, reports on the use of *M. oryzae* and its metabolites to induce *Streptomyces* to produce antibiotics are relatively rare.

In this study, the synthesis of *S. bikiniensis* HD-087 antibiotics was stimulated and enhanced using cell-free filtrates of *M. oryzae* as inducers. The inhibitory effect of *S. bikiniensis* HD-087 metabolites on the growth of *M. oryzae* in the Ind group was significantly higher than that in the Non-ind group. This indicated the feasibility of using target microorganisms and their metabolites as inducers to obtain highly active antibiotics, and the possibility of obtaining antibiotics with stronger targeting properties, which is a good strategy for mining biocontrol agents.

Various organic acids and hormones in *M. oryzae* inducers have been observed to fluctuate markedly at different culture times, a process that involves signaling cascades and cell homeostasis, and can activate or up-regulate the biosynthesis of multiple antibiotics by regulating complex cascades, playing a key regulatory role in the antibiotic production process of *Streptomyces.* The fact that signaling molecules can activate secondary metabolism of *Streptomyces* has been recognized [1]. Among the signal molecules in *M. oryzae* inducers, phosphoric acid had the highest

up-regulation multiplicity. In living organisms, it participates in phosphorylation reactions and plays a regulatory role in many proteins and enzymes in cell signaling pathways [22]. Another notable upregulator in this study was norepinephrine. Which is a multifunctional signaling molecule that can bind to its corresponding receptors, including G protein coupled receptors (GPCRs) [6]. GPCRs sense environmental signals, regulate complex physiological processes, and influence microbial processes such as spore formation, antibiotic production, and morphological differentiation [29]. Moreover, our correlation analysis indicated that these signaling molecules are inextricably linked with each other, with significant correlation and possible direct or indirect mutual responses or interactions that play a non-negligible role in stimulating the secondary metabolite production process of *S. bikiniensis* HD-087.

*M. oryzae* inducers can also influence the synthesis of some potential antimicrobial substances. For example, although there have been no reports on the antibacterial activity of coniferyl acetate, coniferyl alcohol is a specific antifungal substance a precursor of eugenol, which has significant antifungal activity [3]. Based on the toxicity of acids and alcohols, we speculate that coniferyl acetate is an important antifungal substance. Similarly, melanostatin has not been reported to have significant antimicrobial activity, but acts as a melanin inhibitor, affecting the development, resistance, and pathogenicity of various pathogenic fungi, including the formation of appressorium [19, 20, 45]. Whether apigenin-7-sulfate has an antibacterial effect has not been reported, but it is known that apigenin can active apoptosis [20], which hints at the potential antibacterial activity of apigenin-7-sulfate. All these substances have good application potential.

We found that some of the key substances in primary metabolism, especially those associated with the Embden-Meyerhof–Parnas pathway, TCA cycle, amino acid biosynthesis, and fatty acid metabolism pathways, underwent significant changes in the Ind group, such as isocitrate, α-ketoglutarate, succinate, citrate, acetyl-CoA, leucine, isoleucine, lysine, glutamate, proline, tetradecanoic acid, and octanoic acid. These compounds can directly or indirectly influence the synthesis of secondary substances [18]. Zhang et al. [50] found that the desuccinylation process of lysine could stimulate the synthesis of metabolites in *Streptomyces coelicolor* and regulate its morphogenesis. Cheng et al. [8] demonstrated that glutamate and proline are key precursors for the production of streptomycin by *Streptomyces lydicus.* Moreover, leucine, isoleucine, and valine are essential precursors for the synthesis of lipopeptides such as surfactins [16]. In our research, the up-regulation of these amino acids in Ind group suggested that the cell-free filtrate of *M. oryzae* interferes with the secondary metabolism of *S. bikiniensis* HD-087 by affecting its primary metabolism.

At present, it is known that approximately 50–75% of secondary metabolite biosynthetic gene clusters in actinomycetes are associated with the NRPS and PKS pathways [32]. For example, daptomycin and vancomycin are synthesized by NRPS, while PKS is responsible for the synthesis of erythromycin and tetracycline [17, 46]. In *Streptomyces*, NRPS and PKS can also work synergistically to co-synthesize natural products with complex structures and biological activities [4]. Our study showed that the *M. oryzae* inducers significantly stimulated the up-regulation of *nrps* and *pks* antibiotic synthesis gene expression in *S. bikiniensis* HD-087, which is the best annotation for enhancing the antibacterial activity of metabolites in induced culture.

Non-ribosomally lipopeptides, especially iturin and fengycin, can inhibit *M. oryzae*'s mycelial growth [23, 28]. The polyene macrocyclic lactone polyketide compound amphotericin B has broad-spectrum bactericidal activity against fungi such as *Candida*, *Aspergillus*, and *Cryptococcus* [47]. These examples imply that lipopeptides and polyketides can benefit humanity, and the discovery of such new substances has significant social implications. The presence of lipopeptide antibiotics such as surfactin, iturin, and fengycin in the metabolites of *S. bikiniensis* HD-087 was confirmed in our pre-laboratory results [26, 27]. However, in the present paper, we did not focus on analyzing the changes of lipopeptides in strain HD-087 because the molecular weights of most lipopeptide antibiotics exceed 1,000, and metabolomics is more advantageous in analyzing substances with molecular weight below 1,000. This illustrates one of the limitations of metabolomics. However, our previous studies have shown that *M. oryzae* inducers can elevate lipopeptide production. Since the present study aimed to analyze the mechanism of inducing culture to improve antibiotic production, we mainly analyzed intracellular metabolites, and the content of lipopeptides in intracellular substances is very low. We will therefore analyze the influence of inducers on extracellular metabolites in subsequent experiments to consolidate the theoretical basis for the use of cell-free filtrate of *M. oryzae* to induce *Streptomyces* to produce rice-blast-resistant substances.

## Conclusion

The cell-free filtrate of *M. oryzae* improved the ability of *S. bikiniensis* HD-087 to produce antibiotics. The cell-free filtrate of *M. oryzae* cultured for 96 h was rich in a large number of signal molecules and had the best

induction effect, up-regulating the abundance of 16 antimicrobial substances and 20 potential antimicrobial substances as well as the expression of antibiotic synthesis genes *nrps* and *pks* in *S. bikiniensis* HD-087. These results provide an idea for the development of elicitors for *Streptomyces* antibiotic production using the active components in the cell-free filtrate of fungi.

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**Author contributions**
J have made substantial contributions to the conception, the acquisition, analysis, interpretation of data, have drafted the work or substantively revised it , Q analysised, interpretation of data, have prepared Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, C have designed the work, have drafted the work or substantively revised it.

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**Availability of data and materials**
No datasets were generated or analysed during the current study.

## Declarations

**Competing interests**
The authors declare no competing interests.

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