



**Full Length Article**

## Optimization of Anti *Phytophthora infestans* Metabolites Produced by *Myxococcus xanthus* B25-I-1

Zhi Hua Wu<sup>1,2,3</sup>, Pu Yu Zhao<sup>1</sup>, Yi Xiu Ding<sup>1</sup>, Qiang Ma<sup>1</sup>, Xue Han Wang<sup>1</sup> and Hui Rong Liu<sup>\*</sup>

<sup>1</sup>College of Life Sciences, Inner Mongolia Agricultural University, Hohhot 010010, P. R. China

<sup>2</sup>Baotou Teachers' College, Baotou 014030, P. R. China

<sup>3</sup>Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology, Baotou 014030, P. R. China

\*For correspondence: huirong\_liu@imau.edu.cn; wuzhiquaen@163.com

Received 22 August 2020; Accepted 30 December 2020; Published 10 May 2021

### Abstract

This study aimed to screen myxobacteria strains with strong activity against *Phytophthora infestans* and to study its secondary metabolites. Myxobacteria were isolated from a soil sample using the rabbit feces as bait. A strain inhibitive to *P. infestans* was identified by morphological observation and 16S rRNA sequence analysis. The fermentation parameters were optimized through a combination of univariate analysis and orthogonal optimization. The stability and antimicrobial activities of the secondary metabolites were determined using the filter paper method, and the effects on the development of the potato late blight and its pathogen were determined using the *in vitro* leaf method. The myxobacteria B25-I-1 isolated from a soil sample showed strong activity against *P. infestans* and was identified as *Myxococcus xanthus*. The optimal fermentation conditions of *M. xanthus* B25-I-1 were as follows: inoculum size of 10%, shaking at 180 r min<sup>-1</sup>, incubation temperature of 32°C, and incubation time of 8 days. The secondary metabolites were highly tolerant to high temperature, treatment with protease K, and ultraviolet and natural lights. Besides *P. infestans*, the secondary metabolites also showed variant antagonistic effects against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. The results of the potato late blight control on potato leaves indicated that the secondary metabolites could significantly inhibit the infection of detached potato leaves caused by *P. infestans* and caused no damage to the leaves of different varieties of potatoes by themselves. This study provides basic data for the the development of a biopesticide against potato late blight. © 2021 Friends Science Publishers

**Keywords:** Myxobacteria; Potato late blight; *Myxococcus xanthus*; Orthogonal optimization; Secondary metabolite

### Introduction

Myxobacteria are gram-negative bacteria that undergo social behavior, complex multicellular behavior, and morphogenesis (Kim *et al.* 2016), and are higher prokaryotes (Sun *et al.* 2016), known for their predatory lifestyle (Livingstone *et al.* 2018). Myxobacteria are good resources for natural drug screening. With more than 100 core structures and over 500 derivatives been published to date (Herrmann *et al.* 2017). They have joined a large group of microbes with great potential for research and development (Diez *et al.* 2012). Moreover, the biosynthetic potential of myxobacteria is huge, and it is estimated that 50–100% of myxobacteria can synthesize biologically active secondary metabolites (Wenzel and Müller 2009). In prokaryotes ranked according to the number of bioactive secondary metabolites, myxobacteria follow only actinomycetes and bacillus (Wang and Ma 2010), but the fraction of myxobacteria with bacteriostatic activity is

higher than that of actinomycetes. Because myxobacteria can produce several bioactive secondary metabolites with novel structures and unique mechanism of action, they are considered the microfactories of secondary metabolites with biological activity. At present, myxobacteria have become extremely active subjects of domestic and international research (Weissman and Müller 2009).

Potato is the fourth largest food crop worldwide after rice, wheat and maize, and it is the fourth largest staple food in China. Potato late blight is the most serious disease of potato and has seriously hindered the production and industrialization of potato. At present, potato late blight is mainly controlled by chemical agents, and the commonly used agents include metalaxyl mancozeb, metalaxyl, and oxadixyl mancozeb (Wu *et al.* 2020). The extensive use of chemical pesticides damages the environment. Therefore, it is extremely urgent to develop environmentally friend pesticides with high efficiency to control potato late blight.

Potato late blight is caused by *Phytophthora infestans*

(Fry 2008), which belongs to Chromista, Oomycota, Mastigomycotina, Oomycetes, Peronosporales, Pythiaceae. The host range of *P. infestans* is relatively narrow, and *P. infestans* mainly infecting potatoes, tomatoes and other 50 eggplant plants. On these hosts, *P. infestans* usually reproduce by asexual reproduction, and also by sexual oospores in the presence of two mating types, A1 and A2 (Wu 2018). *P. infestans* causes a devastating disease wherein the potato stems and leaves die and the tuber rots, mainly by invading leaves, stems, and potato stalks (Haas *et al.* 2009).

In the present study, a myxobacteria strain (laboratory number for the strain B25-I-1) with significant inhibitory effects on *P. infestans* was isolated from a soil sample collected from the Baotou area, Inner Mongolia Autonomous Region. It was identified by morphological observation and 16S rDNA sequence analysis. The antimicrobial activities of the strain were analyzed, and its fermentation conditions were optimized through orthogonal experiments. The stability and antimicrobial activities of the bioactive secondary metabolites in the concentrated fermentation broth and their anti-disease effects on potato late blight were studied using the *in vitro* leaf method.

## Materials and Methods

### Soil sampling

A soil sample was obtained from grassland in the Baotou area of Inner Mongolia Autonomous Region. It was located at 40°18'38" north latitude and 110°50'32" east longitude and was considered meadow salt soil. Soil was collected 1–10 cm below the surface using the five-point sampling method, and the sample was naturally air dried at room temperature.

### Bacterial and fungal strains

*Staphylococcus aureus* CMCC26003, *Bacillus subtilis* 3-1, *Escherichia coli* DH-5 $\alpha$ , *Saccharomyces cerevisiae* C2-2, *P. infestans* HQK8-3, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* were provided by our laboratory (Applied Environmental Microbiology Laboratory, Inner Mongolia Agricultural University).

### Induction, purification, and preservation of myxobacteria

The soil samples that had been naturally air-dried and screened at 60 mesh were baked at 58°C for 1 h to remove the bacteria with poor heat resistance. Approximately 30 g of the soil sample was placed in a 90-mm Petri dish and soaked overnight at room temperature in a cycloheximide solution at a final concentration of 100  $\mu\text{g mL}^{-1}$  to help remove mold and yeast. The pretreated soil samples were poured into a ST21CX solid culture dish and paved. Half of

sterilized rabbit dung pellets were embedded in the soil sample, and the culture dishes were incubated at 30°C for 6 days. We observed the formation of myxobacteria fruiting bodies over this time (Liu *et al.* 2011).

Myxobacteria were identified and the borders of characteristic colonies or the heads of fruiting bodies were directly picked onto fresh VY/2 medium with a sterile inoculation needle to remove a portion of the larger and immobile bacteria and cultured at 30°C (Guo *et al.* 2007). This method required repeated transfer until there were no other bacteria growing in the medium.

The fruiting bodies of the strains were incubated in the CAS liquid medium and shaken for 36 h before measuring the turbidity in the CAS medium. As myxobacteria grow slowly in nutrient-rich mediums, whereas other miscellaneous bacteria grow relatively fast, the clear CAS liquid culture medium indicates that the myxobacteria strain is pure. The purified myxobacteria strains were stored in 20% sterilized glycerol at –80°C.

### Antagonistic activity of isolated myxobacteria against *P. infestans*

The plate confrontation method (Li *et al.* 2011) was used to detect the antagonistic activity of the strains against *P. infestans*. The *P. infestans* that had been cultivated for 7 days was firstly cultured in rye medium for 3 days. Then, the agar block of the pure myxobacteria after 8 days of culture related to a sterile toothpick to a distance of about 2 cm from the cake of *P. infestans*, and the agar block of sterile VY/2 solid medium was inoculated in the same rye medium as the blank control. The Petri dishes were sealed with a parafilm, and after incubation at 18°C for 7 days, the inhibitory zones were observed.

### Identification of myxobacteria

**Morphological observation:** Referring to Bergey's manual of systematic bacteriology (Boone and Castenholz 2004), the colony morphologies and fruiting bodies of myxobacteria on the VY/2 medium were observed and photographed.

**Molecular identification:** The genomic DNA of strain B25-I-1 was isolated using a kit (Rapid Bacterial Genomic DNA Isolation Kit) from Sangon Biotech (Shanghai) Co., Ltd., China, following the instruction provided by the manufacturer. The set of primers (forward 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 1495R: 5'-CTACGGCTACCTTGTACGA-3') was designed to amplify the partial 16S rDNA of myxobacteria (Zhang *et al.* 2010). The reaction was initiated with denaturation at 94°C for 30 s, followed by primer annealing at 55°C for 45s and primer extension at 72°C for 90 s. After 30 cycles of these parameters, an extension step was performed at 72°C for 5 min. The PCR products were sent to Beijing Liuhe Huada Gene Technology Co. Ltd. for sequencing, and the 16S

rDNA sequences of the tested strains were compared with known sequences in GenBank using the BLAST software.

### Antimicrobial activity of strain B25-I-1

*M. xanthus* B25-I-1 was transferred to fresh VY/2 solid medium and incubated at 30°C for 7 days. The microorganisms *S. aureus*, *E. coli* and *B. subtilis* were individually added into sterilized beef extract peptone liquid medium and shaken overnight. The next day, after centrifugation, the bacterial pellets were evenly distributed on a beef extract peptone solid medium and allowed to dry. *S. cerevisiae* was grown in YPD liquid culture for 18h and then uniformly plated on a potato medium. *M. xanthus* B25-I-1 on the VY/2 solid medium was assembled into microcapsules and inverted onto the beef extract peptone solid medium and potato medium coated with uniform indicator bacteria (Wu *et al.* 2018). The same amount of the VY/2 medium as a blank control was placed on the beef extracts peptone solid medium and potato medium. After incubation for 48 h at 37°C, the antibacterial activities were evaluated.

Using the plate confrontation culture method (Li *et al.* 2011), *F. oxysporum*, *R. solani*, *S. sclerotiorum* and *V. dahliae* were individually inoculated on the fresh PDA solid medium. Subsequently, *M. xanthus* B25-I-1 was assembled into microcapsules and inverted approximately 2.5 cm from each indicator strain, and the VY/2 solid medium as a blank control was inoculated in the same manner. The Petri dishes were sealed with a parafilm, and after incubation at 18°C for 7 days, the inhibitory zones were observed.

### Growth curve of strain B25-I-1

*M. xanthus* B25-I-1 on the solid medium was transferred into 100 mL of the VY/2 liquid medium. After shock culture for 3 days at 30°C and 180 r min<sup>-1</sup>, a sterile rotor with magnetic suspension stirring was used to break the bacterial blocks. The cells of *M. xanthus* B25-I-1 were then transferred to 100 mL of the VY/2 liquid medium at 10% of the inoculum size, and the culture was conducted by shock culture at 30°C and 180 r min<sup>-1</sup>. Cells were collected every 24 h and centrifuged at 8000 r min<sup>-1</sup> for 10 min. The supernatant was discarded and the bacterial pellet was washed with deionized water and centrifuged again at 8000 r min<sup>-1</sup> for 10 min. The supernatant was discarded again, and the bacterial pellet was dried in an oven at 50°C until the weight was constant. Dry weight of the bacteria was measured three times in parallel. The growth curve of *M. xanthus* B25-I-1 was drawn according to the dry weights (Tang *et al.* 2014).

### Localization of bioactive secondary metabolites from strain B25-I-1

Similar to the above process, *M. xanthus* B25-I-1 on the

solid medium was transferred into 100 mL of the VY/2 liquid medium. After shock culture for 3 days at 30°C and 180 r min<sup>-1</sup>, a sterile rotor with magnetic suspension stirring were used to break the bacterial blocks. The cells were then transferred into 100 mL of the VY/2 liquid medium at 10% of the inoculum size, and culture was conducted by shock culture at 30°C and 180 r min<sup>-1</sup> for 7 days. The fermentation broth was centrifuged at 8000 r min<sup>-1</sup> for 20 min to separate the supernatant from the bacterial pellet, and the supernatant was collected. The bacterial pellet was washed three times with sterile water and then disrupted in an ultrasonic cell pulverizer isolation box. The lysed cells were centrifuged again, and the supernatant was collected. The extracellular and intracellular supernatants were lyophilized and weighed, dissolved in sterile water to maintain the same concentration of the extracellular and intracellular substances, filtered through a filtration membrane, and evaluated for the resistance activity against *P. infestans* using the filter paper method.

### Optimization of the fermentation conditions of strain B25-I-1

The cultivation conditions, including inoculum size, fermentation period, culture temperature, and rotating speed of the shaking bed, were optimized through a combination of univariate analysis and orthogonal optimization to provide basic data for future industrial production. Table 1 summarizes the single-factor screening scheme for optimizing the culture conditions.

According to the results of the single-factor experiments, the factors and range of the antagonistic activities of *M. xanthus* B25-I-1 against *P. infestans* were determined, and *M. xanthus* B25-I-1 was then inserted into a seed culture medium at 30°C with 180 r min<sup>-1</sup> shaking for 3 days. The inoculum size was set to 8, 10 or 12%, the fermentation temperature was set to 28, 30 or 32°C, and the fermentation cycle was set to 7, 8, or 9 days. The L9 (3<sup>3</sup>) orthogonal design was used to perform the 3-factor and 3-level orthogonal optimization experiments at 180 r min<sup>-1</sup> shaking. The filter paper method was used to determine the antibacterial activity of the fermentation supernatants.

### Stability of the bioactive secondary metabolites

After fermentation of *M. Xanthus* B25-I-1 under its optimal conditions, the fermentation broth was centrifuged at 8000 r min<sup>-1</sup> for 20 min to separate the supernatant and the bacterial pellet, and the supernatant was collected and lyophilized. The lyophilizate was dissolved in sterile water and filtered through a 0.22- $\mu$ m filter, and the fermentation broth was concentrated 50 times. The filter paper method was used to detect the stability of the concentrated fermentation supernatant against temperature, proteinase K, UV, and natural light, and to determine its capacity for long-term storage.

### Concentration and antimicrobial activities of the bioactive secondary metabolites

The XAD-16 macroporous adsorption resin of 4% was added to the VY/2 liquid fermentation medium, followed by the addition of *M. xanthus* B25-I-1. The resin was collected after fermentation under optimal conditions, and 10 volumes of methanol was added after air-drying under natural conditions. The mixture was eluted three times, and the eluate was combined and concentrated under reduced pressure in a rotary evaporator. After evaporation, the sample was weighed, dissolved in a small amount of methanol, filtered through a 0.22- $\mu\text{m}$  microporous membrane (organic phase), and placed in a refrigerator at 4°C. The antagonistic activities of the bioactive secondary metabolites from *M. xanthus* B25-I-1 against *S. aureus*, *E. coli*, *B. subtilis*, *S. cerevisiae*, *F. oxysporum*, *R. solani*, and *S. sclerotiorum* were evaluated using the filter paper method.

### Antidisease effects of the bioactive secondary metabolites on potato late blight

Four varieties of potatoes (Qingshu No.9, Jizhangshu, Feurita, and Kexin No.1) were used to detect the antidisease effects of the bioactive secondary metabolites. Qingshu No. 9, Jizhangshu, Feurita, and Kexin No. 1 were grown for 5–8 weeks under light conditions. Healthy and similarly sized leaves of mature plants were cut, rinsed with tap water, and placed in sterile 150-mm dishes, the bottoms of which were moisturized with wet sterile cotton wool. The treatment groups had concentrated samples of 200, 100, 10, 5, 3, and 0  $\mu\text{g mL}^{-1}$  applied to the back of the leaves. After air-drying, 100  $\mu\text{L}$  of the *P. infestans* spore suspension was sprayed onto each leaf. The culture dishes were then placed in a 20°C light incubator and cultured for 7–10 days under 16 h light/8 h dark conditions to observe pathogen infection and measure the diseased areas of the leaves. The zoospore suspensions were replaced by sterile distilled water for the control group 1, the methanol concentrate of the bioactive secondary metabolites was replaced with sterile distilled water for the control group 2, and six leaves were used for each treatment. The experiment was performed in triplicate.

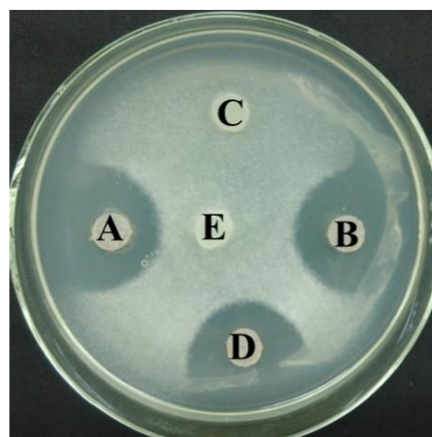
## Results

### Isolation of myxobacteria with antagonistic activity against *P. infestans*

Three strains with the characteristics of myxobacteria were purified from soil samples. All of them showed antagonistic activity against *P. infestans*, and the strain with relatively strong antagonistic activity was named as B25-I-1. The diameter of inhibition zone was about 24 mm. Its activity against *P. infestans* is shown in Fig. 1.

**Table 1:** Screening program of single factors for fermentation conditions

Factors	Levels				
Incubation temperature °C	25	28	30	32	35
Incubation time d	5	6	7	8	9
Inoculum size %	5	8	10	12	15
Rotating speed r min <sup>-1</sup>	150	160	170	180	190



**Fig. 1:** The antibiotic activity of the strain B25-I-1 against *P. infestans*  
(A/B/D) The strain B25-I-1 (C) Blank control (E): *P. infestans*

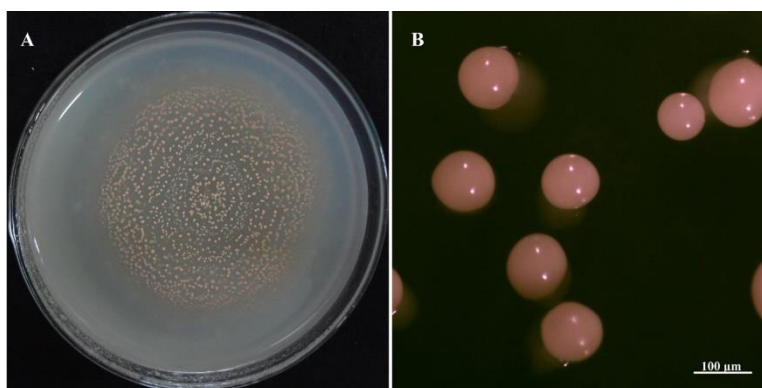
### Identification of the strain B25-I-1

**Morphological identification:** Strain B25-I-1 could form characteristic colonies on the VY/2 medium, with a thin and translucent membrane that was circularly expanded. The fruiting bodies were soft, single-growth, irregularly distributed, yellow, ellipsoidal or spherical and did not have a stalk-like structure but had refraction and transparent mucus on their periphery (Fig. 2). The morphological features of the strain B25-I-1 were similar to those of *Myxococcus xanthus* according to Bergey's manual of systematic bacteriology.

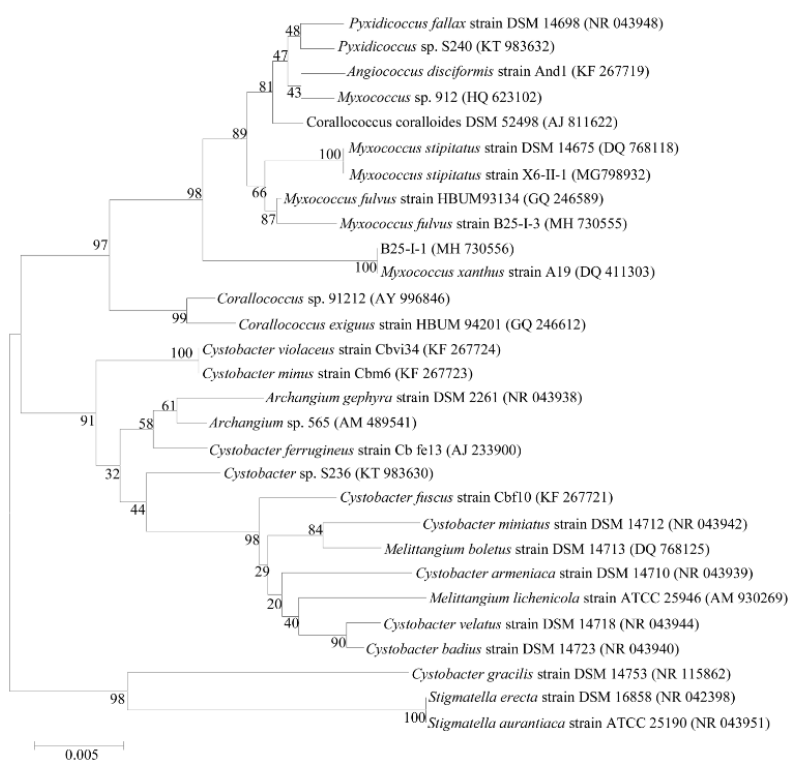
**Molecular identification:** The sequence analysis of the 16S rRNA gene fragment of the strain B25-I-1 was performed, and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 3). The phylogenetic tree indicated that the strain B25-I-1 was *Myxococcus*; it had the highest similarity to the *M. xanthus* A19 (accession number DQ411303), and they clustered in the same phylogenetic evolution branch. Taken together with these morphological characteristics, the strain B25-I-1 was identified as *M. xanthus* (accession number MH730556).

### Antimicrobial spectrum of *M. xanthus* B25-I-1

*M. xanthus* B25-I-1 can kill and dissolve *E. coli* and can inhibit the growth of *B. subtilis*, *F. oxysporum*, *R. solani*, *S. sclerotiorum*, *V. dahliae*, and *P. infestans*. It had the strongest inhibitory activity against *P. infestans* but did not show activity against *S. aureus* or *S. cerevisiae* (Table 2).



**Fig. 2:** The morphological characteristics of the strain B25-I-1  
(A) Colony (B) Fruiting bodies



**Fig. 3:** The phylogenetic tree of the strain B25-I-1 based on the 16S rRNA gene sequence

### Determination of growth curve and distribution of secondary metabolites

As shown in Fig. 4A, the growth of *M. xanthus* B25-I-1 was delayed for 3 days after inoculation, after which the weight of the bacteria began to increase. On the 6<sup>th</sup> day after inoculation, the weight of the cells reached a maximum, then reduced rapidly and stabilized after 7 days. After *M. xanthus* B25-I-1 was fermented and cultured, the filter paper method was used to detect the resistance to *P. infestans* induced by the intracellular and extracellular substances. As shown in Fig. 4B, the extracellular substances of *M. xanthus* B25-I-1 showed an inhibitory effect on the growth of *P.*

*infestans*; however, antagonistic activity of the intracellular material was not detected, thus suggesting that the bioactive secondary metabolites of *M. xanthus* B25-I-1 against *P. infestans* exist outside the cell.

### Optimization of the fermentation conditions

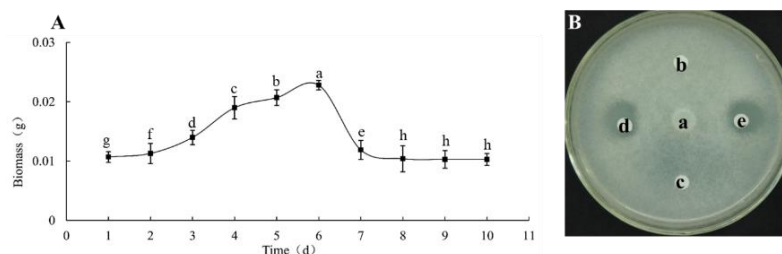
Effects of inoculum size, as shown in Fig. 5A, with an increase in the inoculum size, the antagonistic activity of the fermentation supernatant on *P. infestans* increased initially before decreasing. The antagonistic activity was highest when the inoculum size was 10%, and the diameter of inhibition zone was 12.5 mm. Therefore, in this experiment,



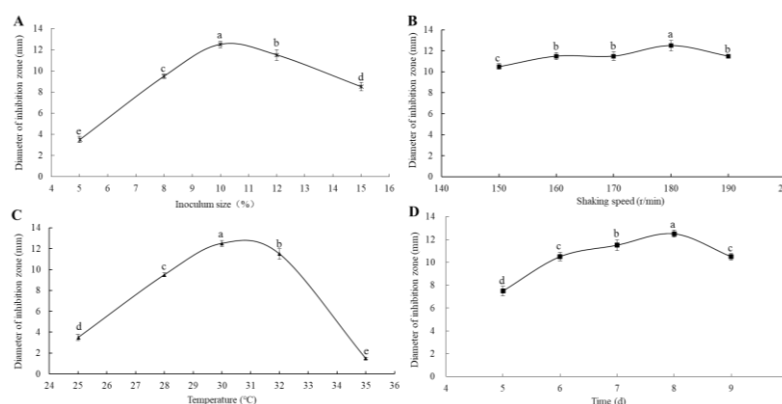
**Table 2:** The antimicrobial spectrum of *M. xanthus* B25-I-1

Indicator microorganism	Antibiotic activity
<i>Escherichia coli</i>	++
<i>Bacillus subtilis</i>	+
<i>Staphylococcus aureus</i>	-
<i>Saccharomyces cerevisiae</i>	-
<i>Fusarium oxysporum</i>	+
<i>Rhizoctonia solani</i>	++
<i>Sclerotinia sclerotiorum</i>	++
<i>Phytophthora infestans</i>	+++
<i>Verticillium dahliae</i>	+

(+++ strong inhibition; (++) medium inhibition; (+) weak inhibition; (-) no inhibition



**Fig. 4:** The growth curve and distribution of the bioactive secondary metabolites of *M. xanthus* B25-I-1 (A) The growth curve (B) distribution of bioactive secondary metabolites (a) *P. infestans* (b) blank control (c) intracellular substances (d/e) extracellular substances. The lowercase letters indicate significant difference level of  $p < 0.05$



**Fig. 5:** Effects of the inoculum size (A), shaking speed (B), incubation temperature (C), and incubation time (D) on the antibiotic activity of the fermentation supernatant. The lowercase letters indicate significant difference level of  $p < 0.05$

the inoculum size of 10% was selected as the optimum inoculum size.

Effects of the shaker speed: During the fermentation of *M. xanthus* B25-I-1, the effects of different rotation speeds on the antagonistic activity of the fermentation supernatant were minimal. As shown in Fig. 5B, the antagonistic activity generally increased over the range of 150–180  $r \text{ min}^{-1}$ , reaching the highest antagonistic activity against *P. infestans* at 180  $r \text{ min}^{-1}$ . At 180–190  $r \text{ min}^{-1}$ , the antagonistic activity was stable; therefore, the optimum speed was determined to be 180  $r \text{ min}^{-1}$ .

Effects of fermentation temperature, as shown in Fig. 5C, the antagonistic activity of the fermentation supernatant increased over a temperature range of 25–30°C. The

antagonistic activity reached maximum at 30°C when the diameter of inhibition zone was 12.5 mm. The antagonistic activity then decreased with a further increase in the temperature, indicating that higher or lower temperatures were not conducive to antagonistic activity. Therefore, 30°C was selected as the optimal fermentation temperature for *M. xanthus* B25-I-1.

Effects of fermentation time, as shown in Fig. 5D, the antagonistic activity of *M. xanthus* B25-I-1 was low after 5 days of fermentation, but the antagonistic activity increased with prolonged fermentation time. The antagonistic activity was maximum on the 8<sup>th</sup> day, and it began to decline by the 9<sup>th</sup> day. Ultimately, the optimal fermentation time was determined to be 8 days.

**Table 3:** Orthogonal experiment design and results for *M. xanthus* B25-I-1

Test number	Factors			Diameter of inhibition zone (mm)
	Temperature (°C)	Incubation time (d)	Inoculum size (%)	Average value
1	28	8	12	10.5d
2	28	9	8	10.9d
3	30	7	12	10.5d
4	30	9	10	11.9c
5	30	8	8	12.1c
6	32	9	12	13.5b
7	32	7	8	13.9b
8	28	7	10	9.5e
9	32	8	10	15.1a
<i>K1</i>	10.3	11.3	12.3	<i>K</i> =12
<i>K2</i>	11.5	12.6	12.2	
<i>K3</i>	14.2	12.1	11.5	
<i>R</i>	3.9	1.3	0.8	

*K1*, *K2* and *K3* indicate the means of the corresponding list, respectively; *R* indicates range; the lowercase letters indicate significant difference level of 0.05

**Table 4:** Variance analysis of orthogonal optimization results for *M. xanthus* B25-I-1

Source of variance	Square sum of the mean squared	Degrees of freedom	Mean square	F	<i>P</i>
Temperature	0.976	2	0.488	15.679	0.060
Incubation time	0.762	2	0.381	12.250	0.075
Inoculum size	0.996	2	0.498	16.000	0.059
Deviation	0.062	2	0.031		

On the basis of the above single-factor experiment results, three factors that had a great influence on the antagonistic activity of the fermentation supernatant of *M. xanthus* B25-I-1 were selected. The inoculum size, fermentation temperature, and fermentation time were orthogonalized. The three factors were selected at three levels for the experimental design. The program and results are shown in Table 3. The fermentation supernatant produced at different temperatures, times, and inoculum sizes had different levels of antagonism to *P. infestans*. The optimum fermentation conditions to produce bioactive secondary metabolites against *P. infestans* were as follows: temperature of 32°C, time of 8 days, and inoculum size of 10%. From the size range analysis, the order of the ability of the supernatant of *M. xanthus* B25-I-1 to antagonize *P. infestans* was temperature > time > inoculum size.

The results using the range calculation cannot be used to quantitatively analyze the error. It was not possible to determine whether the differences between each level of each factor were caused by experimental error or whether there was a substantial difference between the levels of each factor. Therefore, to further reflect the differences among the factors used to optimize the optimal fermentation conditions for *M. xanthus* B25-I-1 to produce substances antagonistic to *P. infestans*, analysis of variance was used to analyze the results, as shown in Table 4. Among the variables, the *P* value for temperature was < 0.05 and close to 0.01, which represented a significant effect on the experimental results. Therefore, temperature was considered the primary factor, and fermentation time and inoculum size were considered secondary factors. Variance analysis was then used to compare the *F* values, and it was further determined that temperature was the main factor influencing the activity of

the fermentation products, and the effects of the fermentation time was slightly greater than those of the inoculum size. Therefore, the effects of the culture temperature, fermentation time, and inoculum size on the activity against *P. infestans* was ordered as temperature > time > inoculation amount, which is consistent with the range analysis results.

#### Stability tests of the bioactive secondary metabolites

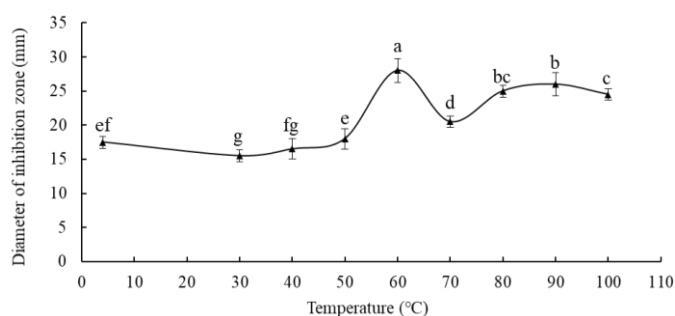
**Thermal stability:** The bioactive secondary metabolites against *P. infestans* maintained high antagonistic activity after being treated for 30 min at temperatures of 30–100°C (Fig. 6). Compared with the antagonistic activity of the bioactive secondary metabolites stored at 4°C, the activities against *P. infestans* were slightly reduced after treatment at 30–50°C for 30 min. However, after treatment for 30 min at 60–100°C, the activities had an increasing tendency. We speculated that the solvent was mainly evaporated due to the higher temperature to increase the concentrations of the bioactive secondary metabolites. It can be preliminarily concluded that temperature has little effect on the activity of secondary metabolites to inhibit the growth of *P. infestans*.

**Proteinase K, UV light, natural light, and storage temperature stability:** The concentrated fermentation broth of *M. xanthus* B25-I-1 was treated with proteinase K for 1 h, UV light for 1 h, and natural light for 1 h and then compared with the concentrated fermentation broth. There was no significant change in the size of the inhibition zone for the growth of *P. infestans* (Fig. 7A). It can be preliminarily indicated that the secondary metabolites were stable to UV light, proteinase K, and natural light, which also suggests that the bioactive secondary metabolites of *M. xanthus* B25-

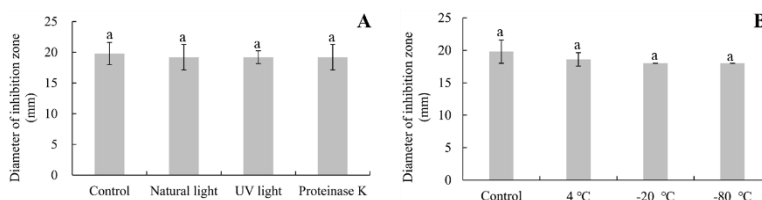
**Table 5:** Antibiotic spectrum of the bioactive secondary metabolites of *M. xanthus* B25-I-1

Indicator microorganism	Antibiotic activity
<i>Escherichiacoli</i>	+
<i>Bacillus subtilis</i>	+
<i>Staphylococcus aureus</i>	+
<i>Saccharomyces cerevisiae</i>	+
<i>Fusarium oxysporum</i>	+
<i>Rhizoctonia solani</i>	+
<i>Sclerotinia sclerotiorum</i>	+

(+) inhibition



**Fig. 6:** Effect of temperature on the antibiotic activity of the metabolites of *M. xanthus* B25-I-1 The lowercase letters indicate significant difference level of p 0.05



**Fig. 7:** Effects of proteinase K, UV light, natural light, and storage temperature on the antibiotic activity of the metabolites. The lowercase letters indicate significant difference level of 0.05

I-against *P. infestans* is not protein based. The activity of the bioactive secondary metabolites against *P. infestans* decreased slightly after storage for 15 days at -80, -20 and 4°C, but the decrease was not significant (Fig. 7B). The results indicated that the secondary metabolites produced by *M. xanthus* B25-I-1 facilitated long-term storage at -80, -20 and 4°C.

### Antimicrobial spectrum of the bioactive secondary metabolites

The secondary metabolites produced by *M. xanthus* B25-I-1 that inhibited the growth of *P. infestans* demonstrated inhibition of the bacteria *E. coli*, *S. aureus*, and *B. subtilis* and fungi *S. cerevisiae*, *F. oxysporum*, *R. solani*, and *S. sclerotiorum* (Table 5). However, *M. xanthus* B25-I-1 showed no activity against *S. aureus* or *S. cerevisiae*. We speculate that the bioactive secondary metabolites secreted by *M. xanthus* B25-I-1 were at low levels, but after the fermentation liquid was concentrated, the concentration of the bioactive secondary metabolites increased, thus showing a broader spectrum of antimicrobial activity than the unconcentrated material.

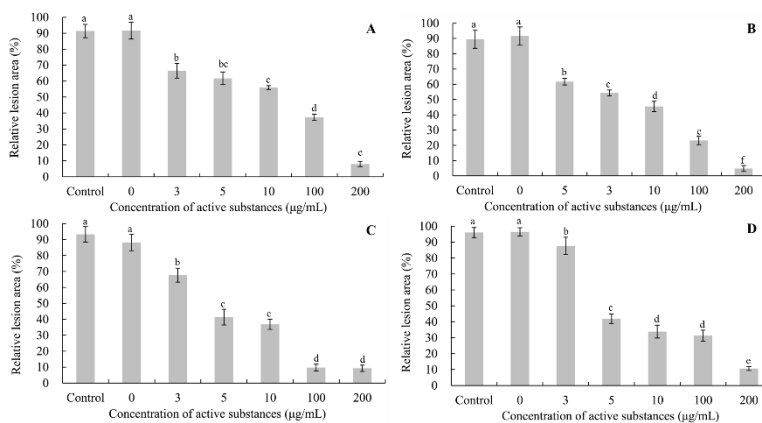
### Antidisease activity of the bioactive secondary metabolites on potato late blight

The relative lesion areas of different varieties of potato leaves were measured using the Adobe Photoshop CS5 software (Cui *et al.* 2009), and the results are shown in Fig. 8. In the control groups treated with only sterile water and only methanol, the percentage of damaged areas of the leaves of the four different varieties of potatoes was over 90% after infection with *P. infestans*. In the test groups treated with different concentrations of the bioactive secondary metabolites from *M. xanthus* B25-I-1, the relative lesion areas of the leaves of the four varieties of potatoes decreased gradually with increasing of concentrations of the bioactive secondary metabolites, indicating that the bioactive secondary metabolites had obvious disease prevention effects on the potato leaves in vitro, and the disease prevention effects were not specific to the variety of the potatoes.

### Discussion

The use of beneficial microorganisms to restrict the harmful microorganisms is an important area of research in the





**Fig. 8:** Effects of the bioactive secondary metabolites on the relative lesion areas of the detached potato leaves (A) Jizhangshu (B) Feurita (C) Kexin No.1 (D) Qingshu No.9. The lowercase letters indicate significant difference level of  $p < 0.05$

development and utilization of microbial resources and is one of the most promising methods to control plant diseases (Jiang *et al.* 2001). In this work, the *M. xanthus* strain B25-I-1 was successfully isolated from a soil sample and showed strong antagonistic activity against *P. infestans*. Abbas *et al.* (2009) isolated a variety of secondary metabolites from *Trichoderma* that significantly inhibited plant pathogens. Caulier *et al.* (2018) found that *B. subtilis* 30B-B6 can also produce resistance to *P. infestans* and can significantly reduce the occurrence of plant diseases. Jiang *et al.* (2010) found that the composite fermentation broth of three strains of actinomycetes against *P. infestans* significantly inhibited the mycelial growth of pathogenic bacteria and caused mycelial deformation or abnormality of sporangia. These studies have shown that there is a great potential to develop a biopesticide against potato late blight from biologically active secondary metabolites produced by microorganisms.

The present study focused on myxobacteria, which are known as the “microfactories” of bioactive secondary metabolites. At present, approximately 600 biologically active substances have been identified from myxobacteria, which have antibacterial, antiviral, anticancer, and thrombolytic activities. These substances represent many novel structures, levels of action, and unique mechanisms of action. For example, chivosazol (Irschik *et al.* 1995) can destroy the integrity and stability of fungal cell walls. Myxochelin (Silakowski *et al.* 2000) interferes with the transport of metal ions from bacteria and some fungi. Disorazol (Khalil *et al.* 2006) acts on tubulin to inhibit cytoskeletal formation. Myxopyronin (Audrey *et al.* 2009), inhibits nucleic acid synthesis primarily by inhibiting bacterial RNA polymerase. These results illustrate the possibility of using myxobacteria to inhibit the growth of *P. infestans*. The secondary metabolites of *M. xanthus* have attracted increasing attention, but little has been reported about the use of its secondary metabolites in the prevention and control of plant diseases, especially potato late blight. In the present study, the *M. xanthus* B25-I-1 showed a strong

antagonistic activity against *P. infestans*, and the bioactive secondary metabolites produced by its fermentation significantly inhibited the infection of detached potato leaves caused by *P. infestans* and caused no damage to the leaves of different potato varieties by themselves. These results complement the existing microbial resources that inhibit the growth of *P. infestans* and provide basic data for the future development of biopesticides against potato late blight.

Although the bioactive secondary metabolites produced by myxobacteria are quite varied (various types, wide resistance, novel structure, *etc.*), factors such as the secretion of mucus and autolysis by extracellular enzymes limit the fermentation and production of bioactive secondary metabolites by these organisms. The optimization of the fermentation conditions mainly includes factors such as fermentation temperature, time, rotation speed, feeding, and ventilation, and these factors generally do not affect the fermentation of the strain independently but interact with each other (Zhang 2011). Xie *et al.* (2017) applied an orthogonal design to optimize the fermentation parameters of *Lactobacillus plantarum* r13 and increased the biomass and effective viable concentration of the bacteria solution. Feng *et al.* (2016) systematically optimized the fermentation and culture conditions of *B. thuringiensis* PanD37 by orthogonal tests, and the L-aspartate  $\alpha$ -deacidase activity of the strain was greatly improved. These findings indicate that the biological activities of microorganisms can be improved by optimizing the fermentation conditions. In the present study, the fermentation conditions of *M. xanthus* B25-I-1 were orthogonally optimized for activity against *P. infestans*. The optimal fermentation conditions were as follows: inoculum size of 10%, shaker speed of  $180 \text{ r min}^{-1}$ , culture temperature of  $32^\circ\text{C}$ , and fermentation time of 8 d. Under the above optimized culture conditions, the antibacterial activity of the fermentation supernatant was determined using the filter paper method and the growth edge of the *P. infestans* was found to be up to 7.3 mm from the filter paper. This work lays the foundation for future developments of biopesticides against potato late blight

based on the *M. xanthus* B25-I-1.

In addition, the present study found that the bioactive secondary metabolites were highly tolerant to high temperature, treatment with protease K, and UV and natural lights and that these substances could be preserved for long periods at a low temperature. These results were consistent with the results for the *M. virescens* YR-35 fermentation broth observed by Ren et al. (2016). However, inhibition of *P. infestans* caused by the bioactive secondary metabolites of YR-35 reached only 15.38% after incubation at 90°C, while a high temperature had little effect on the bioactive secondary metabolites of *M. Xanthus* B25-I-1. The secondary metabolites of *M. fulvus* xt-2 with antipathogenic *P. infestans* activity isolated by Liu et al. (2014) were also sensitive to high temperature. Conversely, the active products of *M. xanthus* B25-I-1 have the potential to control potato late blight at a high temperature found in the fields.

The findings of the present study demonstrated that the secondary metabolites of the *M. xanthus* B25-I-1 have practical value and can be developed and utilized as an effective agent for controlling potato late blight. However, the type of bioactive secondary metabolites, their mechanisms for antagonizing *P. infestans*, and their application in the field still need to be studied further.

## Conclusion

In this study, three strains of myxobacteria were isolated from the soil sample collected from Baotou area of Inner Mongolia Autonomous Region. Among them, the strain B25-I-1 showed strong antagonistic activity against *P. infestans* and was identified as *M. xanthus*. This strain could kill and dissolve *E. coli*, and showed a different degree of inhibition to *B. subtilis*, *F. oxysporum*, *R. solani*, *S. sclerotiorum* and *V. dahlia*. The optimal fermentation conditions for the active substances produced were as follows: inoculum size 10%, shaking speed 180 r min<sup>-1</sup>, incubation temperature 30°C, incubation time 8 days. The active substances were highly tolerant to temperature, the treatment of protease K, ultraviolet light and natural light. The active substances exhibited different degrees of antagonism against *E. coli*, *S. aureus*, *B. subtilis*, *S. cerevisiae*, *F. oxysporum*, *R. solani*, and *S. sclerotiorum*. The active substances could inhibit the infection of the detached potato leaves by *P. infestans* significantly and had no damage to the leaves of different varieties of potato. *M. xanthus* B25-I-1 can produce the active substances against *P. infestans*, and has the potential value of developing biological pesticide against potato late blight. This study provides basic data for the development of biopesticide against potato late blight.

## Acknowledgements

The authors acknowledge the efforts of Ye Dong and Zi Wen Guo for isolating the microorganism.

## Author Contributions

Wu ZH and Liu HR planned the experiments, Wu ZH, Zhao PY and Ding YX interpreted the results, Wu ZH, Ma Q and Wang XH made the write up and Wu ZH statistically analyzed the data and made illustrations.

## Conflict of Interest

The authors declare that they have no conflicts of interest, and manuscript is approved by all authors for publication. There is no conflict of interest among the institutions regarding the research when it has been conducted at the institutions other than authors institutions. If such a conflicting situation arises, the authors will be held responsible.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Ethics Approval

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

## References

- Abbas EH, W Frank, S Jochen, B Heinrich (2009). Detection of viridifungin A and other antifungal metabolites excreted by *Trichoderma harzianum* active against different plant pathogens. *Eur J Plant Pathol* 124:457–470
- Audrey T, G Maxime, B Konstantin, JP Leonetti (2009). Myxopyronin: A punch in the jaws of bacterial RNA polymerase. *Future Microbiol* 4:145–149
- Boone DR, RW Castenholz (2004). *Bergey's Manual of Systematic Bacteriology*. Springer-Verlag, New York, USA
- Caulier S, A Gillis, G Colau, F Licciardi, M Liépin, N Desoignies, P Modrie, A Legreve, J Mahillon, C Bragard (2018). Versatile antagonistic activities of soil-borne *Bacillus* spp. and *Pseudomonas* spp. against *Phytophthora infestans* and other potato pathogens. *Front Microbiol* 9; Article 143
- Cui HW, YL Yang, JT Li, WF Luo, AM Miao, ZX Hu, XN Han (2009). A faster method for measuring relative lesion area on leaves based on software photoshop. *J Anhui Agric Sci* 37:10760–10762
- Diez J, JP Martinez, J Mestres, F Sasse, R Frank, A Meyerhans (2012). Myxobacteria: Natural pharmaceutical factories. *Microb Cell Fact* 30:11–52
- Feng ZB, J Zhang, GZ Chen, YP Cha, JJ Liu, YH Ge, SW Chen, BT Yu (2016). Isolation, identification and fermentation optimization of *Bacillus tequilensis* Pand37 producing L-aspartate  $\alpha$ -decarboxylase. *Acta Microbiol Sin* 56:44–55
- Fry W (2008). *Phytophthora infestans*: The plant (and R gene) destroyer. *Mol Plant Pathol* 9:385–402
- Guo WJ, GJ Tao, WY Tao, FJ Cui, XC Jin, F Bi, ZH Xu, ZH Ao (2007). A myxobacterium strain *Sorangium cellulosum* AHB125 producing epothilone B and other anticancer substances. *Nat Prod Res* 21:1256–1265
- Haas BJ, S Kamoun, MC Zody (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461:393–398

- Herrmann J, AA Fayad, R Müller (2017). Natural products from myxobacteria: Novel metabolites and bioactivities. *Nat Prod Rep* 34:125–160
- Irschik H, R Jansen, K Gerth, G Höfle, H Reichenbach (1995). Chivosazol A, a new inhibitor of eukaryotic organisms isolated from myxobacteria. *J Antibiot* 48:962–966
- Jiang JZ, S Li, HX Wang (2010). Culture condition optimization of three actinomycetes mixed fermentation against *Phytophthora infestans* and mechanism. *J Hebei Univ* 30:78–82
- Jiang JZ, LK Zhao, J Shi, JG Dong (2001). Inhibition of fungal fermented filtrates on *Phytophthora infestans*. *Microbiol Chin* 28:55–59
- Kim YJ, HJ Kim, GW Kim, K Cho, S Takahashi, H Koshino, WG Kim (2016). Isolation of coralmycins A and B, potent anti-gram negative compounds from the myxobacteria *Corallococcus coralloides* M23. *J Nat Prod* 79:2223–2228
- Khalil MW, F Sasse, H Lünsdorf, YA Elnakady, H Reichenbach (2006). Mechanism of action of tubulysin, an antimetabolic peptide from myxobacteria. *Chem Biol Chem* 7:678–683
- Li LY, JZ Jiang, W Guo (2011). Screening of antagonistic bacteria against *Phytophthora infestans* and its inhibition effect. *Plant Dis Pests* 2:49–55
- Liu BH, L Yang, LF Feng, LX He (2011). Isolation and purification of myxobacteria in Chengdu. *Anim Husb Feed Sci* 3:25–27
- Liu PS, N Li, FY Feng, HR Liu (2014). Identification of a myxobacterium strain against *Phytophthora infestans* and its activity analysis. *Sci Technol Eng* 14:40–45
- Livingstone PG, RM Morphew, DE Whitworth (2018). Genome sequencing and pan-genome analysis of 23 *Corallococcus* spp. strains reveal unexpected diversity, with particular plasticity of predatory gene sets. *Front Microbiol* 9; Article 3187
- Ren XB, ZL Zhang, PY Zhao, ZH Wu, HC Cui, HR Liu (2016). Isolation and identification of the strain YR-35 resistant to *Phytophthora infestans* and its metabolites. *Chin J Biol Cont* 32:379–387
- Silakowski B, B Kunze, G Nordsiek, H Blöcker, G Höfle, R Müller (2000). The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15. *Eur J Biochem* 267:6476–6485
- Sun YW, T Tomura, J Sato, T Iizuka, R Fudou, M Ojika (2016). Isolation and biosynthetic analysis of haliamide, a new PKS-NRPS hybrid metabolite from the marine myxobacterium *Haliangium ochraceum*. *Molecules* 21:59–66
- Tang SJ, R Xiao, Y Wen, H Qin, W Liu, XZ Ding, LQ Xia (2014). Isolation and identification of Myxobacteria strain STXZ54 with antitumor activities. *Acta Microbiol Sin* 54:532–542
- Wang DT, ZL Mang (2010). The molecular biology of myxobacterium. *Chem Life* 30:779–782
- Wenzel SC, R Müller (2009). Myxobacteria-'microbial factories' for the production of bioactive secondary metabolites. *Mol Biosyst* 5:567–574
- Weissman KJ, R Müller (2009). A brief tour of myxobacterial secondary metabolism. *Bioorg Med Chem* 17:2121–2136
- Wu ZH (2018). Isolation of Myxobacteria from the central region of inner Mongolia and their activity and components against potato late blight pathogen. *Dissertation*. Inner Mongolia Agricultural University, China
- Wu ZH, PY Zhao, YX Ding, Q Ma, XH Wang, HR Liu (2020). Identification and secondary metabolites of strain B25-I-3 against *Phytophthora infestans*. *Microbiol Chin* 47:3586–3599
- Wu ZH, N Li, XZ Ma, Y Dong, ZW Guo, HR Liu (2018). Diversity and bioactivities of myxobacteria in Daxing'an Mountains. *Microbiol Chin* 45:266–283
- Xie FX, FF Zhang, K Zhou, YJ Zhao, Q Zhao, HB Sun (2017). Isolation, identification and fermentation optimization of lactic acid bacteria for aquaculture water purification. *Acta Microbiol Sin* 57:304–314
- Zhang ZD, YQ Xie, M Chu, MY Gu, SQ Song, QY Tang, J Mao (2010). Study on isolation and identification of endophytes in yam rhizome. *Xinjiang Agric Sci* 47:126–129
- Zhang YT (2011). The studies on isolation, identification and antibacterial activity of *Myxococcus fulvus*. *Dissertation*. Henan University of Technology, China