

Optimization of Fermentation Conditions for Antibiotic Production by Actinomycetes YJ1 Strain against *Sclerotinia sclerotiorum*

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Abstract

In order to improve the productivity of activities substance of actinomycetes YJ1, the effects of the medium components, inoculation volume, medium capacity, rotary speed, fermentation time, temperature and initial pH on the biological activity of YJ1 were investigated by detecting the mycelium growth inhibition rate of *Sclerotinia sclerotiorum*. The results show that the inhibition rate is the highest under the optimum medium composition (g/L), containing sucrose 10.0, soluble starch 10.0, soybean meal 20.0, K₂HPO₄ 0.5, MgSO₄ 0.5, NaCl 1.0 and FeSO₄ 0.01. The maximum antibiotic activity is obtained at the inoculation volume 5%, medium volume in 250 ml flask 75 ml, rotary speed of 180 r/min, fermentation time 4 days, temperature 28°C and initial pH 7.0. The inhibition rate has been increased by 6.71% under the optimized condition.

Keywords: actinomycetes, fermentation, optimization, *Sclerotinia sclerotiorum*

1. Introduction

Exploring biological fungicides from microbial metabolites is an important research direction of fungicides and has been received more attention (NengGong, 2001). Currently, about 1000 varieties of bioactive substances from microorganisms have been discovered, and approximately 2/3 of active substances come from actinomycetes, such as Novobiocin, Zhongshengmycin, etc (Xiao-hei Xing, 1999; Chang-xiong Zhu, 2002; Lei Zhao, 1998; Yue Feng Shi, 2004).

Actinomycetes are important soil microorganisms. They can produce different kinds of secondary metabolites and are the main strains of producing biological active substances. It has great application potential (Dong et al., 2003; Binwang, 2010). Most actinomycetes are heterotrophic and belong to the genus *Streptomyces* which is the largest proportion in the earth (Wei-yan Min et al., 2000; Hua Li, 2007). The mycelium of actinomycetes can elongate and branch unlimitedly. Therefore, actinomycetes mycelia fragment can grow into new mycelium in appropriate conditions by liquid shaking cultivation or industrial fermentation (Ruan-ji Sheng, 1990; Chen, et al., 1999; Elibol, 2002).

The levels of fermentation are related to fermentation time, ventilation, temperature, initial pH etc. Biocontrol strain fermentation is greatly influenced by the combination of media components and culture conditions in laboratory or industrial fermentation. Scientific and rational optimization of fermentation process not only can greatly improve the levels of eventual products, also reduce fermentation costs. Most actinomycetes have a higher requirement for oxygen to grow and optimally produce metabolites. However, medium contained a lot of organic and inorganic substances lead to a low level of dissolved oxygen, therefore, they need proper ventilation conditions to meet the requirements of dissolved oxygen (Qi-rui He, 2010). Fermentation time is a very important factor, which affect the yield and quality of metabolites. Fermentation temperature also affect the growth of actinomycetes. The optimum growth temperature is 23-37 °C for most actinomycetes (Breidt et al., 1995; Stal & Moezelaar, 1997; Spyropoulou et al., 2001; Jian Chen, 2003). The optimum pH is 6.0 to 8.0 and the stable pH is maintained adding appropriate buffer (Lian-xiang Du, 1992).

Actinomycete strain YJ1 was isolated from branches of Ginkgo. It was identified as *Streptomyces felleus* based on morphological, physiological and biochemical characteristics, and the analysis of 16S rDNA sequence (Jia Yao, 2010). It had obvious effects by using the antagonistic actinomycetes to control *S.sclerotiorum* in laboratory, greenhouse and field trials (Jia Yao, 2010). But the suitable fermentation condition has not been reported. In this study, we tried to optimize fermentation condition of *S. felleus* YJ1 and determine the optimal combination of the

medium composition and culture conditions to improve the active ingredient content, reduce costs, enhance the control effect and lay basis in real production application.

2. Materials and Methods

2.1 Organism

Streptomyces felleus YJ1 was isolated from branches of Ginkgo tree and stored in the laboratory. *Sclerotinia sclerotiorum* was isolated from sclerotia formed in the stem of incidence rape from Yaan, China (Wang & Yao 2009).

2.2 Culture Conditions and Medium

Streptomyces felleus YJ1 was maintained on Gause's medium No.1 and *S. sclerotiorum* on PDA. They were stored at 4°C until required.

The medium compositions were as follows:

Gause's medium No.1 contained (g/L): soluble starch 20.0, KNO₃ 1.0, NaCl 0.5, K₂HPO₄ 0.5, MgSO₄·7H₂O 0.5, FeSO₄·7H₂O 0.01, pH 7.2.

A medium contained (g/L): cornstarch 15.0, glucose 10.0, soybean meal 20.0, yeast paste 4.0, NaCl 1, KH₂PO₄ 1, CaCO₃ 3, pH 7.2-7.4.

B medium contained (g/L): glucose 20.0, soybean meal 20.0, peptone 6, NaCl 5, CaCO₃ 4, pH 7.2-7.4.

C medium contained (g/L): cornstarch 30.0, glucose 15.0, soluble starch 10.0, soybean meal 20.0, NH₄Cl 3.0, CaCO₃ 3.0, pH 7.2-7.4.

D medium contained (g/L): soluble starch 5.0, glucose 3.0, soybean meal 5.0, peptone 4.0, yeast paste 4.0, KNO₃ 1.0, NaCl 0.5, CaCO₃ 0.5, KH₂PO₄ 0.5, MgSO₄ 0.5, FeSO₄ 0.01, pH 7.2-7.4.

E medium contained (g/L): millet 10.0, glucose 10.0, peptone 3.0, NaCl 2.0, CaCO₃ 2.0, pH 7.2-7.4.

F medium contained (g/L): sucrose 10.0, soluble starch 10.0, soybean meal 20.0, K₂HPO₄ 0.5, MgSO₄ 0.5, NaCl 1.0, FeSO₄ 0.01, pH 7.2-7.4.

G medium contained (g/L): potato 200.0, glucose 20.0, pH 7.2-7.4.

2.3 Batch Fermentation

Stored strain of YJ1 was inoculated in Gause's medium No.1 and cultivated for 4 days at 28°C in incubator, then intercepted mycelium discs (d=5mm) and inoculated into sterile medium in 250 mL flasks. The flasks were incubated in the dark at 28°C on an eberbach rotary shaker at 180 r.min⁻¹ for 7d. The cultures were centrifuged (8000r, 15 min, 4°C) to separate the Actinomycetes cells and the supernatants. The supernatants were sterilized through 0.22µm bacterial filter and stored at 4°C until required.

2.4 Antibiotic Activity Assay

Antibiotic activity was measured by assaying the mycelium growth inhibition rate of *S. sclerotiorum*. Briefly, 1mL sterile fermentation filtrate mixed with 9 mL PDA was replaced in a dish or without sterile fermentation filtrate as control. After the medium solidifying we inoculated *S. sclerotiorum* mycelium discs which as incubated for 3d on the dish. The dishes were incubated in the dark at 28°C in incubator for 72 h and measured colony diameter of *S. sclerotiorum* using criss-cross method.

$$\text{Inhibition rate} = \frac{\text{Colony diameter of treatment} - \text{Colony diameter of CK}}{\text{Colony diameter of CK}} \times 100\%$$

2.5 Initial Antibiotic Activity Assay

Stored strain of YJ1 fermented under the conditions as follows: medium D, inoculum volume 3%, medium capacity 100mL, temperature 28°C, shaking speed 180 r.min⁻¹, fermentation time 7d. Then the cultures were centrifuged and filtered to assay antibiotic activity.

2.6 Experimental Design

Previous studies indicated that medium, inoculation volume, aeration, time, temperature, agitation rate and initial pH had profound effects on production of antibiotics (Chen et al., 1996, Yang et al., 2001, 2006). Hence, univariate analysis and orthogonal design were used to find the optimized conditions for antibiotic production in flask fermentation experiments.

To determine the effect of medium, time, temperature, agitation rate and initial pH on the productivity of

actinomycetes biocontrol metabolites, YJ1 was cultivated in different parameters as follows: medium (A, B, C, D, E, F and G), time (2, 3, 4, 5, 6, 7, 8, 9 and 10d), temperature (20, 24, 28, 32 and 36°C), agitation rate (60, 100, 140, 180 and 220 rmin⁻¹) and initial pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) using univariate analysis, while inoculum volume (1%, 3%, 5%, and 7%) and medium capacity (50, 75, 100 and 150mL) using L16(4⁵) orthogonal design (Table 1). The experiments were repeated three times.

Table 1. Experimental design of orthogonal design for optimization of inoculum volume and medium capacity

Experiment	Coded value	
	X ₁	X ₂
1	1	1
2	1	2
3	1	3
4	1	4
5	2	1
6	2	2
7	2	3
8	2	4
9	3	1
10	3	2
11	3	3
12	3	4
13	4	1
14	4	2
15	4	3
16	4	4

Data in the table with the same letter means no significant difference

X₁ inoculum volume (1=1%, 2=3%, 3= 5%, 4= 7%)

X₂ medium capacity (1=50, 2=75, 3= 100, 4=150)

3. Results

3.1 Initial Antibiotic Activity

The colony of *S. sclerotiorum* on potato dextrose agar (PDA) with broth was smaller than control. The mycelia growth of *S. sclerotiorum* was significantly inhibited and the antibiotic rate of broth to *S. Sclerotiorum* was 83.98%.

3.2 Effect of Fermentation Medium

In this study the effects of fermentation medium for actinomycetes producing biocontrol metabolites on the mycelial growth of *S. sclerotiorum* are presented in Figure 1. The results showed that the mycelial growth of *S. sclerotiorum* was inhibited. It indicated that YJ1 strains could produce antibiotic metabolites in different fermentation media. However, the production of YJ1 in F medium led to the maximum inhibition of the mycelium growth of *S. Sclerotiorum*. It was about 90.31%. Therefore, F medium was good candidate for YJ1 to produce biocontrol metabolites among the seven fermentation media examined.

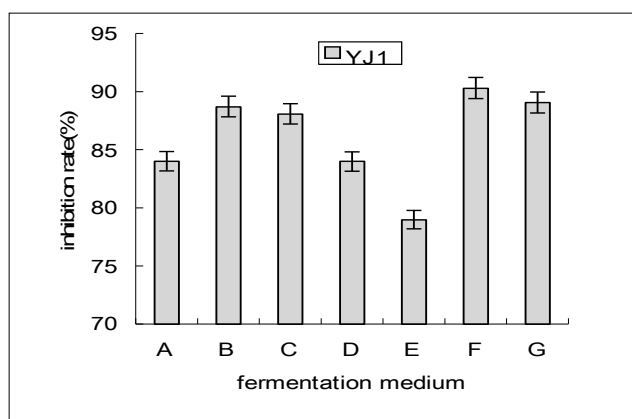


Figure 1. Effects of fermentation medium on the mycelial growth of *S. sclerotiorum*

3.3 Effect of Inoculum Volume and Medium Capacity

Production of antibiotics by *Streptomyces* spp. is generally believed to be an aerobic process. Therefore, dissolved oxygen (DO) is an important factor in the fermentation of *S. felleus* YJ1. In shaken flasks, oxygen supply is related to medium volume. Otherwise, inoculation volume can affect the metabolites accumulation. A lower inoculum density may reduce product formation, whereas a higher inoculum may lead to the poor product formation, especially the large accumulation of toxic substances and also cause the reduction of dissolved oxygen (Mudgetti, 1986).

As seen in Figure 2, the inoculum size and medium capacity appeared to have visible effect on inhibiting the mycelial growth of *S. sclerotiorum*. When the inoculum volume and medium capacity were 5% and 75mL respectively, the inhibition rate reached the maximum (90.92%).

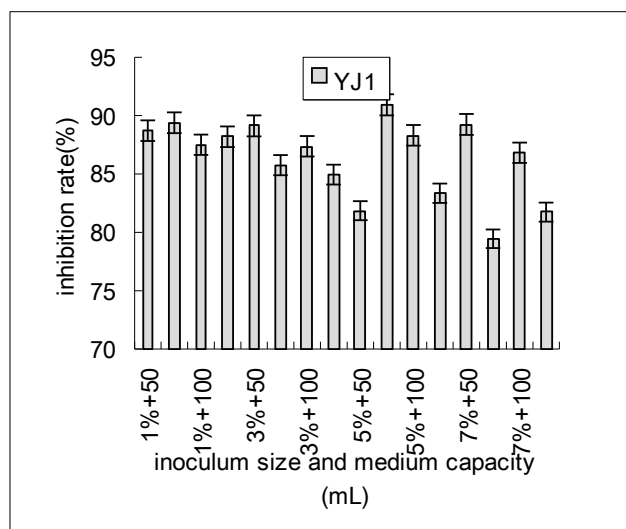


Figure 2. Effects of inoculum volume and medium capacity on the mycelial growth of *S. sclerotiorum*

3.4 Effect of Fermentation Time

Time is also an important factor to affect the fermentation. Increasing time does not mean producing more secondary metabolites. It may produce more toxins to inhibit the production of antimicrobial metabolites.

It can be seen in Figure 3, the fermentation time appeared to have visible effect on the mycelial growth inhibition of *S. sclerotiorum* in 2-4 days. The inhibition rate reached the maximum (90.07%) at 4 days and then decreased slowly. However, there was no obvious difference in 4-10 days. The inhibition rate still remained about 86%.

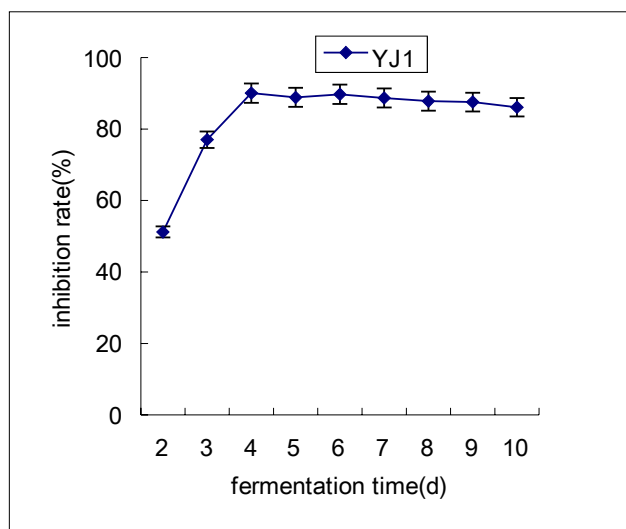


Figure 3. Effects of fermentation time on the mycelial growth of *S. sclerotiorum*

3.5 Effect of Fermentation Temperature

To find out the optimal temperature for actinomycetes producing biocontrol metabolites to inhibit the mycelial growth of *S. sclerotiorum*, YJ1 was cultivated at various temperatures ranging from 20°C to 36°C. As seen in Figure 4, YJ1 strains could produce biocontrol metabolites to inhibit the mycelial growth of *S. sclerotiorum* in 20-36. The inhibitory rate of the mycelial growth of *S. sclerotiorum* increased firstly and then decreased. It might be due to at higher or lower temperature than optimum, the growth of YJ1 was inhibited and further led to the biocontrol metabolites reduction. The maximum inhibitory rate reached 90.24% at 28°C.

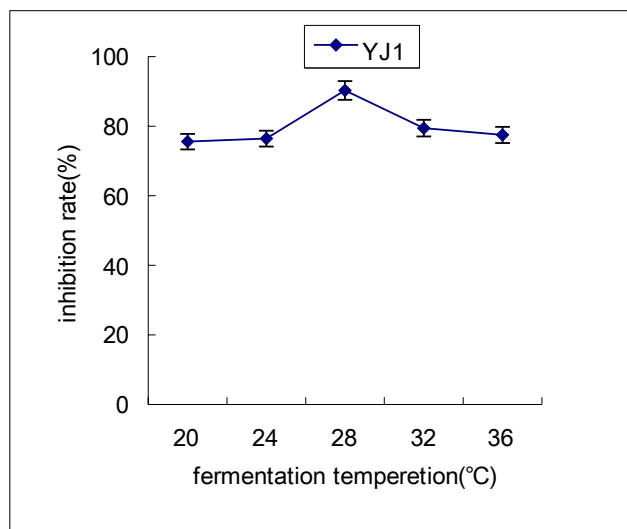


Figure 4. Effects of fermentation temperature on the mycelial growth of *S.sclerotiorum*

3.6 Effect of Fermentation Agitation Rate

Rotary speed can affect oxygen supply. As seen in Figure 5, the inhibition rate of *S. sclerotiorum* mycelial growth showed significant differences under the ranging from 60 to 220 r.min⁻¹. The inhibitory rate increased firstly and then decreased gradually upon increasing the agitation rate. When the speed was 180 r.min⁻¹, the inhibitory rate reached its peak of about 90%.

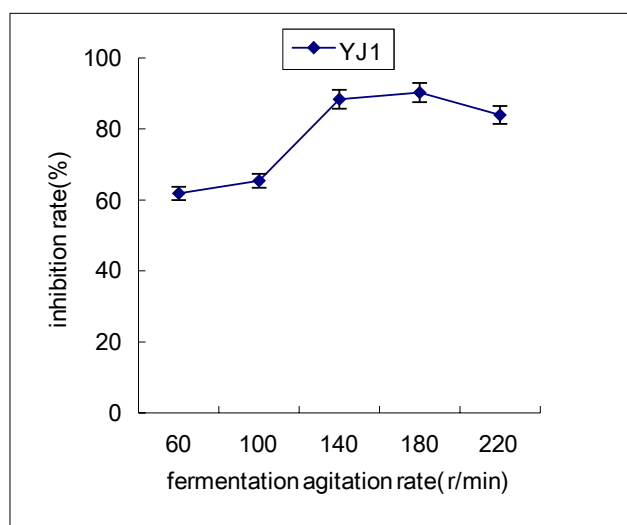


Figure 5. Effects of fermentation agitation rate on the mycelial growth of *S. sclerotiorum*

3.7 Effect of Initial pH of Fermentation Medium

The effects of initial pH on the inhibitory rate of *S. sclerotiorum* are given in Figure 6. It can be seen that the initial pH of fermentation medium had a great impact on the antibacterial activity of YJ1. The antibacterial activity increased with increasing initial pH from 5.0 to 7.0, but any further increase in its values resulted in decreased antibacterial activity. It was due to too high or low initial pH would decrease the production of active substance which affected the inhibitory effect. Under acidic conditions it decreased significantly. When the initial pH was 7.0, the maximum inhibitory rate reached 90.69%. Therefore, the optimal initial pH was around 7.0, which may relate to its natural growing environment.

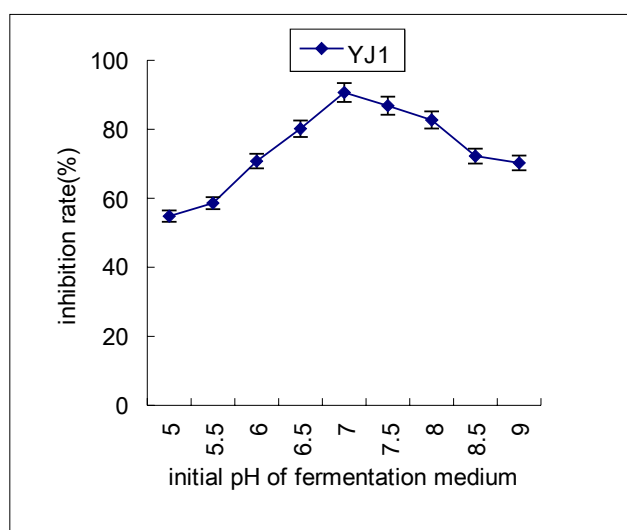


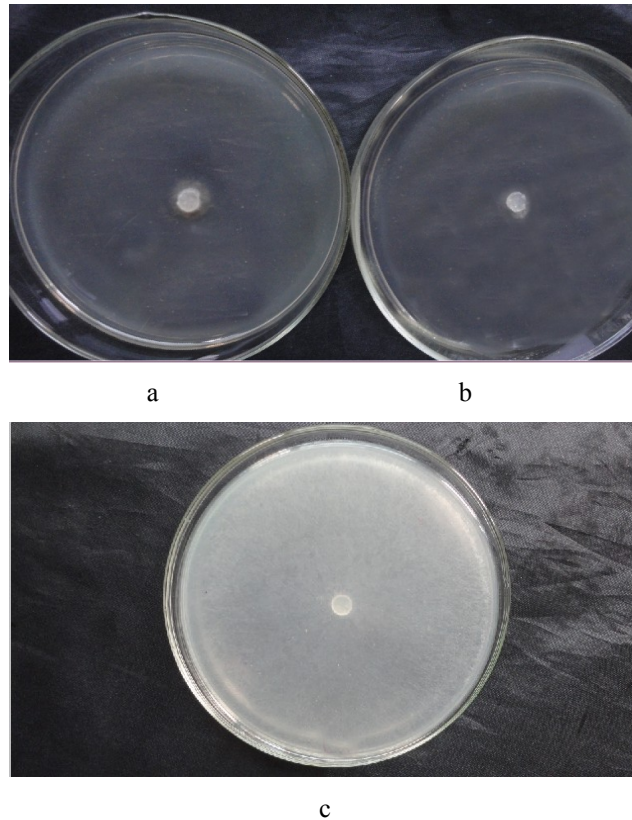
Figure 6. Effects of initial pH of fermentation medium on the mycelial growth of *S.sclerotiorum*

4. Discussion

Actinomycetes fermentation is a complex process, it not only depends on the performance and fermentation medium, also requires the suitable environmental conditions (such as inoculation volume, medium capacity, fermentation time, temperature, agitation rate and initial pH). These factors may affect the antibiotics production. Fermentation has three obvious phases. Firstly, pre-fermentation is cell growth phase, nutrients are gradually consumed and began to produce antibiotics. Secondly, a large number of antibiotics are produced rapidly. Finally, in post-fermentation there is a slow accumulation of metabolites (Jin Wang, 2011). During this stage, actinomycetes even produce toxic metabolites. The reasons may due to cell senescence and autolysis, accumulation of final products or other toxic metabolites which strains can produce in a hostile environment, or the lack of precursors for synthesizing active substances (Liu et al., 2004). Therefore, the medium composition and ratio significantly affected fermentation process. Suitable nutrients can promote the synthesis of metabolites, cell growth, antibiotic fermentation unit, antibiotics extraction process, etc. (Kiers et al., 2000; Palmqvist & Hahn-Hagerdal, 2000).

In this study, we focused on the optimization of nutritional constituents and culture conditions for production of antibiotics by *Streptomyces felleus* YJ1 through Single-factor analysis and orthogonal design to enhance the inhibitory rate of *S. sclerotiorum*. The approach allowed the determination of the culture conditions that gave the highest antibiotics activity for *Streptomyces felleus* YJ1. The better medium of strains YJ1 contained (g/L): sucrose 10.0, soluble starch 10.0, soybean meal 20.0, K_2HPO_4 0.5, $MgSO_4$ 0.5, NaCl 1.0, $FeSO_4$ 0.01, pH 7.2-7.4.

Ideal conditions for fermentation were inoculation volume 5%, medium capacity 75mL, fermentation time 4d, temperature 28°C, agitation rate 180 r.min⁻¹ and initial pH 7.0. In this study, the inhibitory rate had been increased by 6.71% under the optimized condition (Figure 7). The optimization of fermentation process should be considered not only the reduction of the costs of raw material but also the high antimicrobial activity. In this study the information obtained is useful for developing a *Streptomyces felleus* YJ1 cultivation process for efficient production of antibiotics on a large scale.



- a: The PDA mixed with sterile fermentation filtrate which not optimized,
 b: The PDA mixed with sterile fermentation filtrate which optimized,
 c: control.

Figure 7. The colony size of *S.sclerotiorum* on the PDA

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