Optimization of Solid-state Fermentation Process and Development of Biocontrol Agent Based on Cassava Residue from *Streptomyces Microflavus*

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Abstract. In this article, solid-state fermentation of low-cost *Streptomyces microflavus* is studied by using cassava residue, rice husk and other agricultural solid waste resources as the substrate to prepare biopharmaceuticals [1]. The experiment results showed that the *Streptomyces microflavus*’ optimum solid-state fermentation medium and conditions includes the following components: cassava residue 89.85%, rice husk 10%, KH₂PO₄ 0.05%, NaCl 0.05%, MgSO₄ 0.05%. The fermentation temperature is controlled at about 30°C, the initial water content is around 50%, initial pH is 6.6-7, the material thickness is 5cm, the inoculation amount is 7.5%, the cultivation time is 4 days, and the spore yield is 4.8*10⁹ cfu/g. The survival rate of *Streptomyces microflavus*’ spores is 75%, which compared with blank conditions increased by 30% after low temperature vacuum drying. The work of this paper provides some theoretical guidance for the large-scale industrial production of *Streptomyces*, which has valuable application in agricultural field.

Introduction

With the enhancement of people's awareness of ecology, environment and health, the requirement for agricultural sustainable development is more and more urgent for efficient and safe biological pesticide and biological control of crop diseases[2]. Therefore, the biological control of plant diseases has developed rapidly, and the development, production and application of biocontrol agents and biopesticides have drawn more and more attention. Biocontrol agents are an important tool for the comprehensive treatment of plant diseases, which have the unique advantages of safety, non-toxicity, long lasting effect and environmental protection. Accordingly, they provide feasible solutions for environmental pollution, food safety and drug resistance [3-9]. With the development of green agriculture, biological fertilizers has begun to be used on a large scale [10]. Actinomycetes have always been the focus of research as the main biocontrol microbiology. *Streptomyces* is the main source of actinomycetes in agriculture. They can not only inhibit the growth of many pathogenic microbiology, but also stimulate the germination and rooting of crops[10]. Various products can be developed and applied, such as antibacterial, insecticidal, weeding, growth regulating, Anti-virus [11-16].

*Streptomyces* are majority produced by short shelf life liquid fermentation[17], whereas solid-state fermentation has the advantages of low cost, easy to transport and preservation[18]. In this study, solid-state fermentation of *Streptomyces microflavus* was carried out based on solid wastes such as cassava residue and rice husk and the optimum conditions of fermentation and preservation were determined, which laid the foundation for large-scale production of *Streptomyces microflavus*.

Material and Methods

The materials and methods are listed as follows in this study.
Material
S14YB130AK097-type incubator shaker (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.), GNP-9160 water insulated constant temperature incubator (Shanghai Jinghong Experimental Equipment Co., Ltd.), FW-CJ-2FD super Clean Bench (Zhejiang Sujing Purification Equipment Co., Ltd.), BSM320.3 electronic balance (Shanghai Zhuo Jing Electronic Technology Co., Ltd.), GI54DWS high pressure sterilizer (Xiamen Zealway Instrument INC.), NIKON50I optical microscope (Shanghai Precision Instrument Co., Ltd.), XW-80A whirlpool oscillator (Shanghai Jingke Branch Industrial Co., Ltd.)

Slant Culture
Streptomyces microflavus AMYa-008 (provided by Agricultural Culture Collection of China and preserved in Shandong Provincial Key Laboratory of Microbial Engineering) was used in this aritical. The glycerol tube culture strain of Streptomyces microflavus AMYa-008 was inoculated into Gaoshi No. 1 culture, which cultured at 30 °C within 5 d, activated by continuous transfer 2-3 times, placed in 4 °C refrigerator for later use.

Preparation of Seed Liquid
In the ultra-clean platform, picking 1-2 ring bacteria activated by inoculation loop were inoculated into 100 mL SNB medium (sucrose 1.5%, glycerol 1.5%, peptone 4%, potassium dihydrogen phosphate 0.05%, calcium carbonate 0.05%, 0.05% sodium chloride, 0.05% magnesium sulfate, and each component was added in mass fraction) at 500 mL Erlenmeyer flask and, which incubated at 180 r/min on a shaker at 30°C for 4 days. The bacteria activity by plate count reached $3 \times 10^9$ cfu/mL.

Optimization of Material Preparation and Sterilization Process
Cassava residue (50 ~ 80 mesh), rice husk, corn flour, wheat bran were treated with dry and wet heat sterilization respectively.

Wet heat sterilization method: The pH and water content are adjusted after mixing the material, and then sterilized under the condition of 1x10^5 Pa for last 1 h to prepare for using.

Dry heat sterilization method: The material is dried to make the moisture less than 20%, which drying or stir fry 3-5 h at the temperature of 121°C to prepare for using by adjusting the water and pH.

Optimization of Fermentation Formula
Taking cassava residue as substrate, 5% of rice husk, corn flour, wheat bran and urea were respectively selected as different nutritional adjuvants to carry out opitional experiment of different carbon and nitrogen sources.

Optimization of Solid-State Fermentation Process
The fermentation conditions were optimized with cassava residue as the main matrix, rice husk as auxiliary material and compounded by 19:1, and adding 0.05% KH₂PO₄, 0.05% NaCl, 0.05% MgSO₄. The single-factor optimization experimental design of flipping frequency, water content, fermentation temperature, fermentation period, material thickness and inoculum size is shown in Table 1.
Table 1. Optimum conditions for solid-state fermentation of *Streptomyces microflavus*.

<table>
<thead>
<tr>
<th>Number</th>
<th>Condition Optimization conditions</th>
<th>Optimization project</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30°C, 50% water, pH 7, 5 cm, 4d, 5% inoculation not flip, 12h, 24h, 36h, 48h</td>
<td>flipping frequency</td>
</tr>
<tr>
<td>2</td>
<td>not flip, 30°C, pH 7, 5 cm, 4d, 5% inoculation 35%, 40%, 45%, 50%, 55%, 60%</td>
<td>water content</td>
</tr>
<tr>
<td>3</td>
<td>not flip, 50% water, pH 7, 5 cm, 4d, 5% inoculation fermentation temperature 22°C, 25°C, 28°C, 30°C, 32°C, 35°C</td>
<td>fermentation temperature</td>
</tr>
<tr>
<td>4</td>
<td>not flip, 30°C, 50% water, pH 7, 5 cm, 5% inoculation fermentation period 3 d, 4 d, 5 d, 6 d, 7 d</td>
<td>fermentation period</td>
</tr>
<tr>
<td>5</td>
<td>not flip, 30°C, 50% water, pH 7, 4 d, 5% inoculation material thickness 3 cm, 5 cm, 7 cm, 10 cm</td>
<td>material thickness</td>
</tr>
<tr>
<td>6</td>
<td>not flip, 30°C, 50% water, pH 7, 5 cm, 4d inoculum size 2.5%, 5.0%, 7.5%, 10.0%</td>
<td>inoculum size</td>
</tr>
</tbody>
</table>

Orthogonal Experiment of Solid-State Fermentation Conditions

The effects of different content of rice husk, material thickness, initial water content and initial pH on fermentation level and fermentation process were investigated using cassava residue as substrate, rice husk as auxiliary material, inoculum volume 5% and fermentation 4 days at about 30°C.

Trial Enlargement of Solid-State Fermentation and Optimization of Its Conditions

Using the above single factor experiments and orthogonal test conditions optimized for fermentation pilot scale amplification, the optimization of *Streptomyces* fermentation by three different ways (shallow plate fermentation, nest reactor fermentation and ventilation and fermentation), select the optimal fermentation mode.

Fermentation Level and Parameter Detection

Growth curve of *Streptomyces microflavus* AMYa-008: The above optimized fermentation process was used for solid fermentation, pH and water content of the samples were measured every 12 hours and the number of viable bacteria was determined by the plate count at the rate of 85% or more mature spore.

PH measurement: Sampling in the solid-state fermentation medium by five sampling method, weighed 5g into the flask containing 45 mL tap water, shake well and measured by pH meter[^19].

Determination of water content: As above the method, weighed 10g into a clean glass plate, 105°C baked 2 hours to measure the moisture content[^19].

Actinomycete spores production count: Mixing material at the end of the fermentation, taking 10 g samples in 90 mL sterile saline containing glass beads, the gradient dilution was made after shaking 30 min, then coating for plate count and the viable bacteria content in the solid matrix was determined[^20].

Low-Temperature and Drying Storage Technology

At the end of fermentation, the material was dried under vacuum at 4-6°C and stored at low temperature below 10% water content. After 1 and 3 months, the changes of spores of *Streptomyces microflavus* AMYa-008 were determined.
Results and Discussion

Figure 1. Mycelium and spores from Streptomyces microflavus AMYa-008 (×1000).

Comparison of Two Sterilization Processes

Table 2. Comparison of dry and wet heat sterilization process.

<table>
<thead>
<tr>
<th>Sterilization method</th>
<th>the number of bacteria after sterilization (X10^9 cfu/g)</th>
<th>Fermentation yield (X10^9 cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry heat sterilization</td>
<td>0.03</td>
<td>3.6</td>
</tr>
<tr>
<td>Wet heat sterilization</td>
<td>0.02</td>
<td>3.6</td>
</tr>
</tbody>
</table>

The above experimental results shown that the average fermentation yield of both sterilization methods can reach 3.6 × 10^9 cfu/g. Meanwhile, the dry and wet heat sterilization processes have almost no effect on the final fermentation yield. Its advantages include the convenience to develop large-scale production and the relatively low cost. Therefore, the dry heat sterilization process is used to sterilize.

Optimization of Fermentation Formula

According to the above experimental results, it can be seen that the cassava residue as substrate, adding 5% rice husk to cassava residue as the nutritional supplement is the best solid-state fermentation formula, which is superior to other raw materials, and the fermentation yield is 4.2×10^9 cfu/g. While carrying out fermentation adding nothing, corn flour, wheat bran, urea as the auxiliary materials, the bacteria content was 3.6×10^9 cfu/g, 3.0×10^9 cfu/g, 2.8×10^9 cfu/g, 3.5×10^9 cfu/g respectively. After fermentation, the content of the viable bacteria in cassava residue was 16.7% higher than that of blank, which was 40%, 50%, 20% higher than those of the above respectively. It is likely that the reason for this result is that it is possible to provide sufficient air for
the strain by the supporting effect of rice husk. Because corn flour and wheat bran are too rich in nutrition, the risk of bacterial infection is too high. Bacterial infection led to the fermentation yield is lower than the blank, so it is not suitable for fermentation of this strain.

Effects of Turning Frequency on the Fermentation Yield of *Streptomyces Microflavus* Amya-008

![Figure 3. Effects of different turnover frequency on fermentation yield.](image)

Based on the above experimental results, it is concluded that the highest fermentation level of the not flip condition can reach 4.2×10⁹ cfu/g. With the increase of turning frequency, the chances of streptomycete mycelium rupture increased, and the possibility of contamination was also increased. Therefore, the turning conditions were finally selected as not to be turned.

Optimization of Temperature, Water Content, Inoculation and Material Thickness

![Figure 4. Effects of different temperature, water content, and inoculation and material thickness on fermentation yield.](image)
According to the above experimental results, the optimized conditions were as follows: the fermentation temperature was 28~32°C, the initial water content was 50%, the inoculation was 7.5%, and the material thickness was 5 cm. Based on the cost consideration, 5% inoculation can be selected if industrial enlargement is carried out.

Comparing the fermentation results at different fermentation temperatures, the fermentation production reached the highest level of $4.2 \times 10^9$ cfu/g at 30°C, which was respectively increased by 68%, 82.6% than the fermentation level of fermentation temperature at 25°C, 35°C. And under the same conditions, the fermentation level reached $5 \times 10^8$ cfu/g at 22°C, $4.1 \times 10^9$ cfu/g at 28°C and $4.05 \times 10^9$ cfu/g at 32°C.

Comparing the fermentation results of 35%, 40%, 45%, 50%, 55% and 60% water content, the highest level of fermentation of *Streptomyces microflavus* is $4.2 \times 10^9$ cfu/g when the water content is 50%. Under the same conditions, the fermentation level increased by 16.7% than 45% water content ($3.6 \times 10^9$ cfu/g) and 68% than 55% moisture content ($2.5 \times 10^9$ cfu/g).

Comparing the fermentation results of 2.5%, 5%, 7.5%, 10% inoculation, the highest level of fermentation of *Streptomyces microflavus* is $4.5 \times 10^9$ cfu/g when the inoculation is 7.5%. Under the same conditions, the fermentation level increased by 125% than 2.5% inoculation ($2.0 \times 10^9$ cfu/g), 71.4% than 5% inoculation ($4.2 \times 10^9$ cfu/g) and 46.5% than 10% inoculation ($4.3 \times 10^9$ cfu/g).

Comparing the fermentation results at different material thickness, the fermentation production reached the highest level of $4.2 \times 10^9$ cfu/g at 5cm, which was respectively increased by 31.25%, 50% and 110% than the fermentation level of material thickness at 3cm, 7cm and 10cm.

**Effects of Different Fermentation Period on the Fermentation Yield of the *Streptomyces Microflavus***

![Figure 5. Effects of different fermentation cycles on fermentation yield.](image)

**Orthogonal Optimization Test of Solid Fermentation Conditions**

Orthogonal test was further conducted on the amount of rice husk, material thickness, water content and pH that greatly affected the growth of the target bacteria. The results are shown in Table 3. From the table 3 range analysis, we can see that the order of influence of 4 factors on the biomass of the target bacteria is pH > material thickness > husk content > water content. It is pH that has the greatest influence on the number of living bacteria, and the water content has the least effect on the results. Through the comparison of the number of live bacteria at different levels from various factors, the best conditions for solid fermentation were as follows: the added amount of rice husk was 10%, the material thickness was 5 cm, and the initial water content was 50% and the initial pH7. At this time, the optimized fermentation density reached $4.8 \times 10^9$ cfu/g, which increased 33.3% compared with the initial condition.
Table 3. Orthogonal experiment design and results.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Factor</th>
<th>Live bacteria (×10⁸ cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rice husk addition</td>
<td>Material thickness (cm)</td>
</tr>
<tr>
<td>1</td>
<td>5%</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5%</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5%</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>10%</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>10%</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>10%</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>20%</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>20%</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>20%</td>
<td>7</td>
</tr>
<tr>
<td>K1</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>K2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td>22.7</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>7.3</td>
<td>9</td>
</tr>
</tbody>
</table>

Note: K1, K2 and K3 respectively represent the average of the sum of the corresponding levels of each factor, R represents the range of the average of corresponding levels of each factor.

Pilot Scale Enlargement and Condition Optimization Results of Solid-State Fermentation

Figure 6. Effects of different fermentation methods on fermentation yield.

The experimental results showed that the amount of sporulation for solid fermentation pilot scale enlargement in the tray fermentation was the highest, reached to 4*10⁹ cfu/g. Due to material accumulation thickness is too large, resulting in material permeability is poor and affecting the strain respiration, sporulation of wound heap fermentation is lower, which had only 1.8*10⁹ cfu/g. The low sporulation rate of ventilation fermentation is due to continuous water supplement and turnover during fermentation process. Like this, it is easy to introduce heterozygous bacteria, then seize the target strain site and inhibit its growth, resulting in high heterozygous bacteria rate and low fermentation level, which had only 1.5 * 10⁹ cfu/g.
Water Content, Temperature and Ph Trends

The whole process of *Streptomyces microflavus* AMYa-008 solid fermentation which was designed in this paper period about 4 days, the strain in the first two days propagate fast for the long mycelium in the logarithmic growth phase, which began to form long chain at 1-3 days, and collapse into a single spore at 2-4 days. Meanwhile, the pH value increased from 6.8 to 7.8 and the water content decline from 50% to 48% due to the rapid growth in 1-3 days of *Streptomyces microflavus*, the growth came into stable period and began to form spores after 2-3 days, the fermentation ended after 4 days.

**Spore Preservation of Streptomyces Microflavus Amya-008**

Cryogenic vacuum drying, compared with conventional drying or no drying, the survival rate of spores respectively increased by 10% and 25% at one month, and the survival rate of spores respectively increased by 15% and 30% at three months. Therefore, the low-temperature vacuum drying was chosen as the after treatment preservation process.

<table>
<thead>
<tr>
<th>Test time</th>
<th>Blank</th>
<th>Ordinary drying vacuum drying</th>
<th>Low-temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>One month</td>
<td>60</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>Three months</td>
<td>45</td>
<td>60</td>
<td>75</td>
</tr>
</tbody>
</table>

**Conclusion**

The results showed that the best medium formula and conditions of solid fermentation: cassava residue 89.85%, rice husk 10%, KH₂PO₄ 0.05%, NaCl 0.05%, MgSO₄ 0.05%. The fermentation temperature of the strain was 30 °C, the thickness of the material was 5 cm, the inoculum size was 7.5%, the culture time was 4 days, the spore yield was 4.8*10⁹ cfu/g, which increased 33.3% compared with the initial blank condition. After the pilot scale amplification, the sporulation quantity was 4.5*10⁹ cfu/g. Taking into account the cost of production, spore yield was 4.2*10⁹ cfu/g when the inoculum size was 5%, and 4.0*10⁹ cfu/g after amplification. The surviving rate of
spores was the highest, reaching 75% by the low temperature vacuum drying process after the fermentation of *Streptomyces microflavus* AMYa-008. This study initially explored the solid fermentation process and spore preservation method of *Streptomyces microflavus* AMYa-008, which made it capable of sporulation and preservation for a longer time. This laid the foundation for the future development of *Streptomyces microflavus* into agricultural biocontrol agents [21].

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