The Solid Fermentation State’s Optimization of Trichoderma Harzianum M1

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Abstract. In order to exploitation a solid inoculant of Trichoderma harzianum M1 which has a significant effect on Gramineae pasture, so that it can be better in the production of the application, the solid-state fermentation substrate was optimized in this experiment. The optimal combination of the solid-state fermentation of Trichoderma harzianum M1 was screened by single factor and orthogonal experiment. The effects of carbon, nitrogen and inorganic salt on the sporulation quantity of Trichoderma harzianum M1 were further studied. The results showed that: The optimal substrate for the solid-state fermentation of Trichoderma harzianum was the ratio of grass powder: wheat bran: rice bran was 3: 2: 1, the optimal carbon source was glucose, the optimal nitrogen source was peptone, and the optimal inorganic salt was dipotassium hydrogen phosphate. Therefore, the optimal formula of Trichoderma harzianum M1 solid-state fermentation substrate was as follows: the ratio of grass powder: wheat bran: rice bran was 3: 2: 1, glucose 1%, peptone 0.05%, dipotassium hydrogen phosphate 0.01%, the number of sporulation up to 5.9×10^9 cfu/g.

1. Introduction
Spore preparations are the main form of Trichoderma agricultural production applications. How to obtain a large number of Trichoderma spores is a key technology for preparing Trichoderma spore preparations. Solid-state fermentation is recognized as one of the best ways to obtain fungal spores, not only with high yield, but also with good spore quality.1-3 However, there are few reports on the application of solid fermentation of Trichoderma harzianum to pasture. Therefore, the Trichoderma strain M1, which has been significantly promoted in the previous period, has a significant growth promoting effect on the fermentation substrate, carbon source, nitrogen source and inorganic salt, and is excellent for promoting Trichoderma in the pasture. Provide a basis for the application of high yield and quality cultivation.
2. Orthogonal combined solid matrix screening

In order to further determine the ratio of each component of the solid fermentation medium, the three substrates selected by the above experiment were 3 factors, each factor was set to 3 levels according to different mass ratios, and the number of spores was used as the standard according to the orthogonal design. Formulated into 9 formulas for optimal combination matrix screening, the fourth column is set as a blank column. The specific treatment methods are shown in Table 1. Each of the 500 ml cones was filled with 40 g of base and 55 ml of water, sealed with a sealing film, and killed at 20 °C for 20 min. Under sterile conditions, add 5 ml of spore suspension to the above 9 solid mediums, stir well, incubate in a constant temperature incubator at 28 °C, shake once a day, sample after 7 days of fermentation, count with blood cells. The plate counts the spore concentration. The test was repeated 6 times. The formulation of the solid fermenting medium is determined based on the results of the orthogonal composition.

Table 1 Design of the orthogonal experiment for solid-state fermentation

| Recipe number | Grass powder | Wheat bran | Rice bran | Blank column |
|---------------|--------------|------------|-----------|--------------|
| 1             | 1            | 1          | 1         | 1            |
| 2             | 1            | 2          | 2         | 2            |
| 3             | 1            | 3          | 3         | 3            |
| 4             | 2            | 1          | 2         | 3            |
| 5             | 2            | 2          | 3         | 1            |
| 6             | 2            | 3          | 1         | 2            |
| 7             | 3            | 1          | 3         | 2            |
| 8             | 3            | 2          | 2         | 3            |
| 9             | 3            | 3          | 1         | 1            |

3. Effect of additives on solid fermentation

Glucose, sucrose, starch, L-glutamic acid, maltose, glycerol, and fructose were added to 1% by mass of the solid medium; the medium conditions were the same as in 2.1.3.2, and the samples were sampled at 7 days of fermentation, and the spore concentration was counted using a hemocytometer. The test was repeated 6 times. The most common source of spores was selected.

On the basis of adding carbon source, KNO₃, peptone, yeast extract, ammonium nitrate, NH₄Cl, ammonium sulfate and urea were added according to 0.5% of the mass of solid medium; the medium condition was the same as 2.1.3.3, and the optimal carbon source was added for fermentation for 7 days. When sampling, the spore concentration was counted using a hemocytometer. The test was repeated 6 times. The most suitable source of spores was selected.

Adding a carbon source and a nitrogen source, respectively, adding manganese sulfate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, calcium chloride, sodium dihydrogen phosphate, magnesium sulfate, potassium chloride according to 0.01% by mass of the solid medium; The base conditions were the same as in 2.1.3.4, and the optimal nitrogen source was added for 7 days fermentation, and the spore concentration was counted using a hemocytometer. The test was repeated 6 times. The most common inorganic salt was selected.

4. Test results

Since the optimal combination medium A₃B₂C₁ derived from range analysis and analysis of variance was not present in the orthogonal table, a validation test was performed on this optimal combination. The average number of spores obtained by this optimal combination was $3.26 \times 10^9$ / g, which was not significantly different from the maximum $3.18 \times 10^9$ / g in the orthogonal table. Considering that the cost of grass powder (Huangzhucao) is low in production practice, it is determined that the best combination A₃B₂C₁ from the range analysis and variance analysis is the optimal fermentation...
substrate for this experiment, namely grass powder: wheat bran: The ratio of rice bran is 3:2:1.

**Table 2** Analysis of the orthogonal experiment for solid-state fermentation

| Test number | A (Grass powder) | B (Wheat bran) | C (Rice bran) | Sporulation (10^9 /g) |
|-------------|------------------|----------------|---------------|----------------------|
|             | X1               | X2             | X3            |                      |
| 1           | 1                | 1              | 1             | 2.7                  |
| 2           | 1                | 2              | 2             | 2.8                  |
| 3           | 1                | 3              | 3             | 1.5                  |
| 4           | 2                | 1              | 2             | 0.9                  |
| 5           | 2                | 2              | 3             | 1.8                  |
| 6           | 2                | 3              | 1             | 2.3                  |
| 7           | 3                | 1              | 3             | 1.4                  |
| 8           | 3                | 2              | 1             | 2.6                  |
| 9           | 3                | 3              | 2             | 3                    |

| K1 | 7.5 | 5.6 | 9.3 |
| K2 | 6.0 | 9.2 | 8.8 |
| K3 | 7.8 | 8.9 | 5.5 |
| k1 | 2.51 | 1.85 | 3.11 |
| k2 | 1.98 | 3.07 | 2.93 |
| k3 | 3.39 | 2.96 | 1.84 |
| R  | 1.40 | 1.22 | 1.27 |

**Fig 1** The relationships of factors and yield of mycelium biomass
Table 3 The orthogonal experiment results of solid-state fermentation by variance analysis

| Source of variation | SS          | df | MS          | F            |
|---------------------|-------------|----|-------------|--------------|
| Grass powder (A)    | 9.021422222 | 2  | 4.510711111 | 44.42267289**|
| Wheat bran (B)      | 8.182488889 | 2  | 4.091244444 | 40.29165451**|
| Rice bran (C)       | 5.9144      | 2  | 2.9572      | 29.12328567**|
| Model error         | 8.467822222 | 2  | 4.233911111 | 41.69667348  |
| Test error          | 1.827733333 | 18 | 0.101540741 |              |
| Total               | 33.413866726 |    |             |              |

5. Effect of adding carbon source on solid fermentation
The number of spores of Trichoderma harzianum M 1 added with glutamic acid in the solid fermented medium was the highest, and the most spores of Trichoderma harzianum in the secondary culture medium were glucose. Glutamic acid plays an important role in the process of protein metabolism in the organism, and involves many important chemical reactions in the micro-organisms. At the same time, amino acid is the growth factor required for the growth of micro-organisms, M. harzianum M 1 can achieve good growth on this matrix. Glucose is a carbon source that can be used directly by Trichoderma, and therefore its base can promote the growth and reproduction of M. harzianum M 1. Compared with the control, the number of spores added to the sucrose, starch, maltose and fructose is reduced because they can produce glucose after they are decomposed, and the micro-organisms need to be further decomposed before they can be used. In the industrial production of fungicides, the cost of production is considered, and the cost of glutamic acid is too high. Therefore, glucose is selected when nitrogen is added. The results of this study also showed that the addition of maltose to the culture medium had the few sporulations, which may be due to the different concentrations of added maltose.

6. Effect of adding nitrogen source on solid fermentation
After adding egg white mash in the culture medium, M. harzianum M 1 had the highest sporulation, followed by ammonium sulphate and yeast extract. The least sporulation was urea, and the difference in sporulation of ammonium chloride with ammonium nitrate and potassium nitrate was not significant. The egg white is rich in animal protein, plant protein, micro-protein and other substances. These substances can provide micro-organisms with carbon sources, nitrogen sources, growth factors and other nutrients, which are beneficial to microspore spore production. The subject also found that the number of spores of Trichoderma harzianum added to the culture medium was significantly higher than that of the potassium nitrate and ammonium nitrate added to the culture medium. This is consistent with the study of D a r n i e l s o n et al. When Trichoderma grows in the presence of a carbohydrate as a medium, it acts as a nitrogen source and the ammonia salt is better than the nitrate. After the addition of urea in the medium, the number of spores of Trichoderma harzianum M 1 was the least, which was consistent with some studies. The growth and sporulation of Trichoderma mycelia decreased with the increase of urea dosage, that is, urea was not conducive to the growth and sporulation of Trichoderma mycelium. Some people have found that ammonium nitrogen can greatly increase the germination rate of spores of Trichoderma spr 0 1 strain, while nitrate nitrogen does not promote the spore germination of this strain, but the results of this study indicate that chlorine is added to the medium. The difference in the number of ammonium and potassium nitrate spores is not significant. The reason may be that the concentration of ammonium chloride added in this experiment is low, which results in the promotion of ammonium nitrogen to sporulation of Trichoderma.

7. Effect of adding inorganic salts on solid fermentation
After the addition of inorganic salts in the medium, the number of spores of Trichoderma was significantly higher than that of the control group. The highest number of spores was dipotassium hydrogen phosphate, followed by sodium dihydrogen phosphate, potassium chloride, potassium
dihydrogen phosphate, but four The difference in the number of spores produced by inorganic salts was not significant. The number of spores produced after the addition of inorganic salt was higher than that of the control group. The addition of inorganic salts to the solid fermented medium could affect the number of spores of Trichoderma. This is consistent with research reports that mineral elements (such as Cu, Zn, Fe, K, Mg, Ca, etc.) can affect the growth and sporulation of Trichoderma. Inorganic ions play an important role in the solidification process. It has a variety of functions such as adjusting the solid-state acid-base balance, maintaining the osmotic pressure, and constructing an auxiliary substance such as a proton or a functional group of the protein. The three inorganic salts with the highest sporulation in this study contained K+, which was consistent with Liu Shilun’s research. K+ and Fe2+ promoted the sporulation of Trichoderma strain Tv04-2. Wang Yongdong et al. found that the effect of phosphate on the sporulation was better than that of the compound salt. This paper finds that potassium dihydrogen phosphate is the best inorganic salt of M. harzianum M1. Potassium hydrogen phosphite not only provides phosphorus, but also potassium, which is beneficial to the growth of Trichoderma harzianum.

8. Single solid matrix screening
In addition to hemicellulose, cellulose and starch, wheat bran also contains a variety of metal ions and vitamins, which can provide a carbon source for the growth of microorganisms, including biotin and B vitamins, as well as magnesium, phosphorus, iron. Metal ions such as calcium are essential growth factors for microorganisms, and wheat bran has good ventilation and heat dissipation, which is beneficial to microbial sporulation. Grass powder is made of Huangzhucao. Grass powder is rich in crude fiber, vitamins and various metal ions. These factors are beneficial to the growth of microorganisms. At the same time, the looseness of grass powder can loosen the medium and facilitate microbial production. spore. Rice bran contains a large amount of crude protein and crude fiber, which can provide a large amount of protein and carbohydrates for microorganisms, which is beneficial to the sporulation of Trichoderma harzianum. However, rice bran mixed with water to form a dough, which is poor in gas permeability and is not conducive to the growth of microorganisms. The number of spores produced by rice bran is second only to grass flour and wheat bran. Although the bean meal is rich in protein, there are many anti-nutrient factors at the same time. For example, the trypsin inhibitor and soy coagulin can cause some microorganisms to not grow well on the substrate. The nutrient composition of corn flour is 8% crude protein, 2% crude fiber, 0.66% calcium, and 0.26% phosphorus. The crude protein content is only half of wheat bran, and the content of carbohydrate and inorganic salt is very small. And corn flour has poor gas permeability and heat dissipation, which is not conducive to the growth of microorganisms. Rapeseed cake contains a lot of protein and carbohydrates, containing mineral elements such as calcium, phosphorus and magnesium. However, many anti-nutritional factors in rapeseed cake are not conducive to the growth of microorganisms. For example, phytic acid will reduce calcium, phosphorus and magnesium. Utilization, tannins can be combined with protein to significantly reduce their nutritional value. Sulfur glucoside is a major harmful toxic substance. These anti-nutritional substances cause Trichoderma to not sporulate on its substrate.

Conclusion
The results of this study show that: a). The optimal medium for solid fermentation of Trichoderma harzianum M1 is grass powder: wheat bran: rice bran is 3:2:1, the optimized carbon source of Trichoderma harzianum M1 solid fermentation medium is glucose, the solid fermentation medium of Trichoderma harzianum M1 The optimized nitrogen source is peptone, and the optimized inorganic salt of Trichoderma harzianum M1 solid fermentation medium is dipotassium hydrogen phosphate. b). The optimized formula of Trichoderma harzianum M1 solid fermentation medium is: grass powder: wheat bran: rice bran is compatible with 3:2:1 ratio, glucose 1%, peptone 0.05%, dipotassium hydrogen phosphate 0.01%, sporulation amount up to 5.9×10^9/g.
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