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Research Article

Optimization of Process Conditions of Monascus Seed Koji by Orthogonal Test Design

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Abstract: On the basis of single-factor test, we conducted optimization study on the three major factors (material moisture content, cultivation time, temperature) that affect the production of purebred Monascus spores through orthogonal trial. The results show that, in the trial-level range, moisture content of materials and cultivation time have larger effect on the test results, while the cultivation temperature has little effect. The optimum conditions in preparation of purebred Monascus seed koji are: 60% moisture content of materials, 5 d cultivation time, 35°C cultivation temperature, 0.1 mL inoculation, 10 g filling and 0.2% lactic acid. To verify this test scheme, ultimately the number of spores produced by Monascus is 3.35×10^7/g.

Keywords: Monascus, process optimization, purebred mould starter, spores production conditions

INTRODUCTION

Monascus is small filamentous saprophytic fungi, belongs to *Eumycophyta*, *Ascomycotina*, *Plectomycetes*, *Eurotiales*, *Monascaceae* (Chulee, 2002; Wei and Wang, 2008). It often exists in fresh grass, soil, rubber, dried fish, sediment on river and pine root tissues (Wei and Wang, 2008). It's widely used in food coloration, wine, corrosion and fields of medicine (Lizuka and Lin, 1981). Currently, Monascus has been used in liquor brewing process with achievements, such as improving the esterification of Daqu. Zhuang (2005) reported Monascus in liquor production can play a role in reducing lactic acid and ethyl lactate, while producing esterified synthase which induces acid and ethanol to generate ethyl caproate, that's very favorable for the flavor and quality of the fermentation liquor (Liu *et al*., 2008; Jian *et al*., 2001). Wuhan Jiacheng Biotechnology Co., Ltd. cooperating with Sichuan Longchang DiTan Jiao Co., Ltd, also found that adding 0.5% monascus in strengthen daqu production, the esterifying power improved 3.8 mg/g, 16.8%, compared with the original process (Zhang and Ma, 2007). But in actual process, due to the relatively weak fecundity and slow growth of Monascus, compared to other fungi, it's very difficult to incubate, with no competitive advantage in the large Monascus system; it is difficult to form the dominant species (Wei and Wang, 2008; Xing and Chen, 2001). Therefore, it is expected to achieve better results using a seed with higher order of magnitude to make Monascus species maintain a growth advantage, through pure culture techniques and expanding cultivation.

Seed koji culture conditions have some influence on koji quality. Koji quality can not only directly affect the quality of a finished koji and also affect the utilization of raw materials and production rate (Wu, 2011). In solid-state culture condition, to produce Monascus majorly reproduce by asexual or sexual spores through aerial hyphae, hyphae can also reproduce. The number of spores also has some relevance with the total hyphae (Wei and Wang, 2008). In addition, studies show changes in the number of spores also associated with the amount of enzyme in a certain extent, in this way, using spores as koji quality assessment is practically significance. However, there is little relevant literature (Leng, 2004).

In this study, we used 250 mL cork triangle flask with tampon, bran as raw material, Monascus spore count as an indicator, to determine the scope of its optimum level through single-factor test, combined with orthogonal experiment to optimized the main factors of purebred Monascus sporulation, in order to get a kind of Monascus seed koji with larger number and stable quality of spores and providing helps for the mechanized production of koji.

MATERIALS AND METHODS

Materials and reagents: Monascus, offered by Zhejiang University of Technology; bran, commercially available, water content 14.6%; glucose (AR), offered by Chengdu Kelong Chemical Reagent Factory; lactic acid (AR), offered by Chengdu Kelong Chemical Reagent Factory.

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Table 1: Factors and levels of the test

Inoculum (mL)	Incubation time (d)	Filling capacity (g)	Material moisture (%)	Lactic acid (%)	Glucose (%)	Cultivation temperature (°C)
0.1	3	5.0	30	0	0	29
0.6	4	7.5	40	0.2	1	31
1.1	5	10.0	50	0.4	2	33
1.6	6	12.5	60	0.6	3	35
2.1	7	15.0	70	0.8	4	37

Notes: Material moisture in wet basis

Table 2: Orthogonal test program and result analysis

Number	A (Material moisture)	B (Cultivation temperature)	C (Incubation time)	D (Blank)	Spores /*10^7/g
1	1 (40%)	1 (31°C)	1 (4d)	1	0.71
2	1	2 (33°C)	2 (5d)	2	1.28
3	1	3 (35°C)	3 (6d)	3	1.43
4	2 (50%)	1	3	2	1.93
5	2	2	1	3	2.06
6	2	3	2	1	3.02
7	3 (60%)	1	2	3	2.35
8	3	2	3	1	3.12
9	3	3	1	2	2.36
k1	1.14	1.67	1.71	2.28	
k2	2.34	2.15	2.22	1.86	
k3	2.61	2.27	2.16	1.95	
R	1.47	0.60	0.51	0.42	

Instruments and equipment: Constant temperature incubator (LHP-250) from Changzhou Putian Instrument Manufacturing Co., Ltd.; biological microscope (BH200) from Ningbo Sunny Instruments Co., Ltd.; temperature steam sterilizer (SYQ-DSX-280B) from Shenan Medical Devices; e-balance (6102) from Hangzhou Youheng Weighing Equipment Co., Ltd.; Refrigerator (BCD-256L) from Qingdao Leader Electrical Appliance Co., Ltd.; super clean workbench: Suzhou Antai Airtech Co., Ltd.

Test methods:

- **Process flow:** Process flow is shown as follows (Fig. 1).
- **Preparation of bacterial suspension:** Take a slant tube with cultured Monascus, aseptically, add 10 mL of sterile saline, gently scraped spores on the ramps with inoculation loop, fully shaking, making a spore suspension with suitable concentration, inoculate in the spore suspension.
- **Spore count:** Sample dilution: Weigh accurately Koji 1.00 g, pour into 250 mL conical flask with glass beads, add 95% ethanol 5 mL, sterile water 20 mL, dilute sulfuric acid 10 mL, sufficiently shaking, to disperse all conidia, then filter by multilayer gauze, wash (the filtered residue exclude spores), dilute to 250 mL. Slide preparation: Take one drip dilution with a straw to the computing grid on the blood count board and then press the cover slip gently from side to side, making the cover sheet and count board completely seal, without bubbles in the counting region, blot the excess spill spore suspension with filter paper, stand for a few minutes to settle the spores. Observation and counting: Observation with low magnification lens or high magnification lens. Spores in dilutions were at different spatial locations on blood count board and can be seen in a different focal length. Therefore, it must be counted by the mobilization of micro screw, in order not to make omissions. Spores often located on the two-lane of a large grid, two sides should always be taken in counting and the other two sides should be given up, in order to avoid double counting. This test uses a 25×16-based counting board, takes spores among the upper left, lower left, upper right, lower right and the middle, totally five major divisions, twice repeated in each sample and the average is the number of spores for the sample:

$$\text{Spores (a/g)} = N/80\times4\times10^6\times n$$

where,
N : Total number of spores within 80 small grids
a : Number of spores
n : Dilution factor

Single factor trial design: Referred to relevant literature and combined with pre-experiment, it's found out that bran is more suitable than rice for the preparation of Monascus Koji. Secondly, the main factor affecting Monascus sporulation conditions and the scope of this study are shown in Table 1. In every single factor, the other factor is fixed: inoculum 0.1 mL, incubation time 5d, filling 10 g, material moisture content 50%, lactic acid 0.5%, glucose 0% and cultivation temperature 33°C.

Orthogonal test design (Li and Hu, 2012): Orthogonal test is most commonly used trial design method. On the basis of single-factor test, using Monascus sporulation as indicators and material moisture, incubation time, cultivation temperature as independent variables, to optimize the process conditions of purebred Monascus Koji sporulation. Test levels of each factor were shown in Table 2.

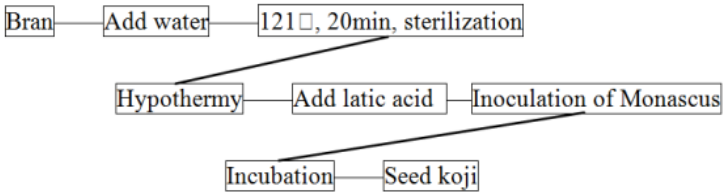

Fig. 1: Process of koji making

RESULTS AND DISCUSSION

Effects of Inoculation on Monascus sporulation: Figure 2 show the inoculation amount changed in the range of 0.1 mL~2.1 mL, while the number of spores changed little, showing a slight decline. When the inoculation amount was 0.1 mL, the spores reached a maximum of 1.65×10^7/g, with the increasing of the inoculation amount, the number of spores reduced gradually. Generally, the larger the amount of inoculation, the faster the growth of the microorganisms, but when inoculation was excessive, nutrient competition occurred between the individual microorganism, the growth of cells would be adversely affected. Though, it is not true less inoculation was better, it was observed that increasing the amount of inoculation to 0.01 mL, the number of spores was significantly lower than that in other levels.

Effects of incubation time on Monascus sporulation: Figure 3 shows, when the incubation time was 3d~7d, the number of spores increased with increased and then decreased, in day 4, sporulation reached a maximum of 2.1×10^7/g, with the continued extension of the incubation time, the number of spores reduced gradually and later began to increase. This could be the accumulation reaches a maximum with the cultivation and then some of the spores began to germinate, reducing the sporulation.

Effects of filling capacity on Monascus sporulation: Figure 4 shows, when filling capacity was between 5~15 g, the number of spores increases with the filling capacity. The number of spores reached a maximum of 1.93×10^7/g when loading capacity was 10 g, followed by a slight decrease. Filling capacity has more important effect on Monascus in the flask. Mainly reflected that, the filling capacity is too small, while space utilization is low and the moisturizing effect is poor, which is not conducive to the growth of Monascus. High filling capacity does not help heat dissipation and oxygen supply in flask, such a closed environment.

Effects of moisture content on Monascus sporulation: Figure 5 shows that, when the moisture content is between 30 to 70%, the number of spores increased and then decreased, the maximum number is 1.83×10^7/g when moisture content is 50%. The moisture content of the bran has a significant impact on

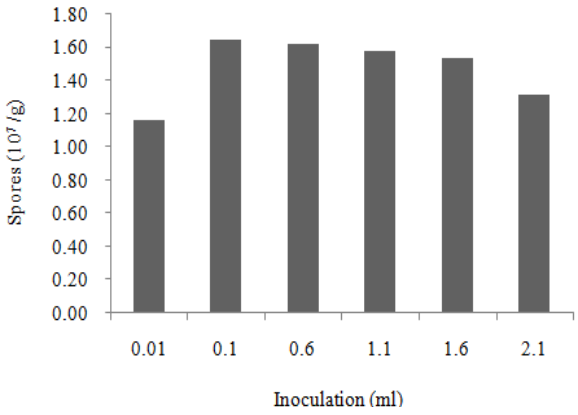

Fig. 2: Effects of inoculation on Monascus sporulation

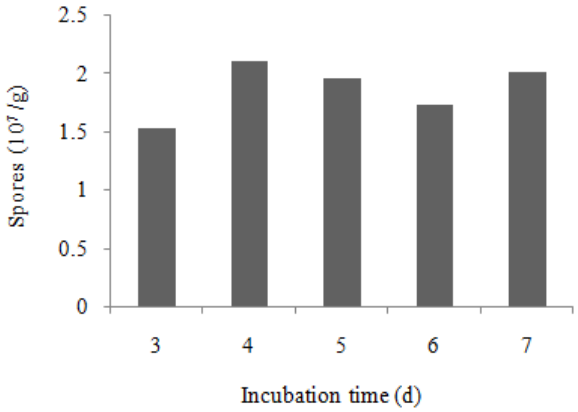

Fig. 3: Effects of incubation time on Monascus sporulation

Monascus sporulation, too much water or too little could not be conducive to sporulation. The reason may be that too little water is not conducive to the growth of Monascus, while too much water is not conducive to oxygen supply.

Effects of lactic acid mount on Monascus sporulation: Figure 6 shows that, when the amount of lactic acid was between 0 to 0.8%, Monascus sporulation increased and then reduced. A maximum sporulation was 2.34×10^7/g when 0.2% lactic acid was added. Monascus is kind of acidophilus bacteria (ability PH 3.5). Adding a small amount of lactic acid into bran medium can enhance Monascus sporulation (Guo and Yang, 2003).

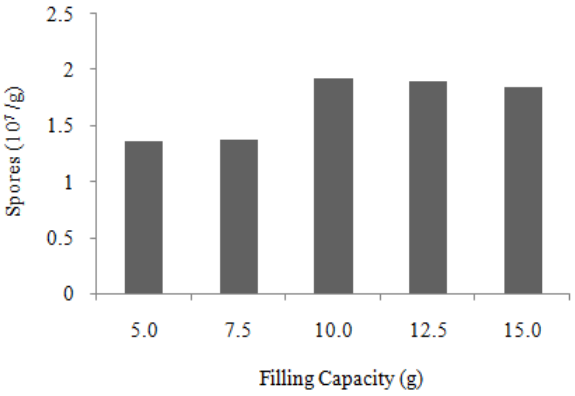

Fig. 4: Effects of filling capacity on Monascus mouldstarter sporulation

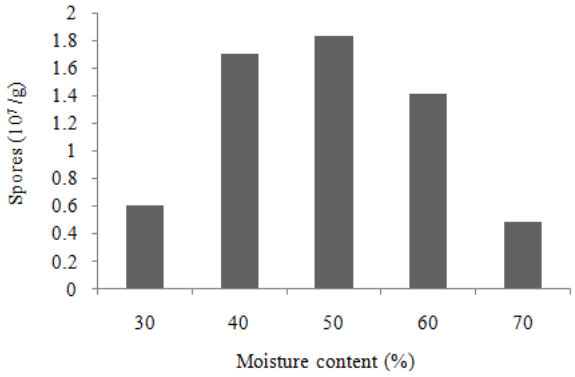

Fig. 5: Effects of moisture content on Monascus koji sporulation

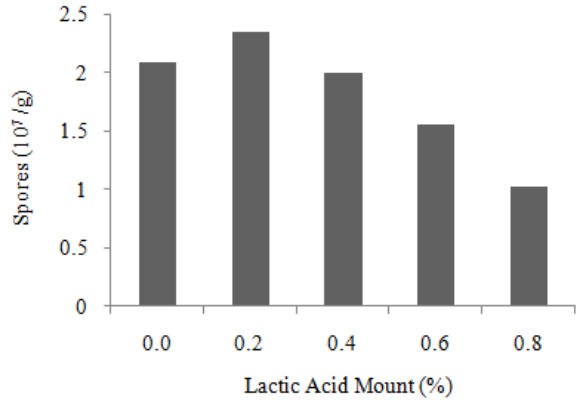

Fig. 6: Effects of lactic acid amount on Monascus mouldstarter sporulation

Effects of glucose amount on Monascus sporulation: Figure 7 shows that, Monascus sporulation was more without addition of glucose, while Monascus sporulation decreased with the increase in the amount of glucose. It has been reported that, a certain amount of glucose do help to improve esterification ability of Monascus (Zhang and Ge, 2012, 2009). These inconsistent results in the koji process may caused by

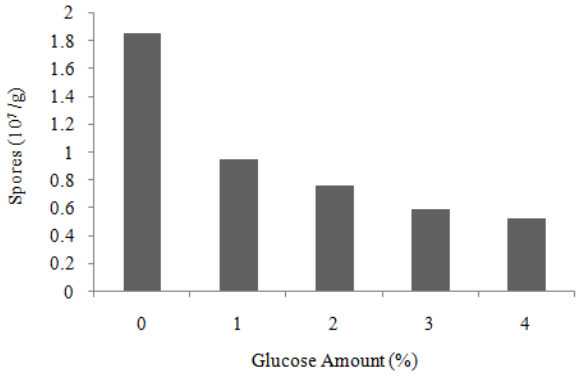

Fig. 7: Effects of glucose amount on Monascus mouldstarter sporulation

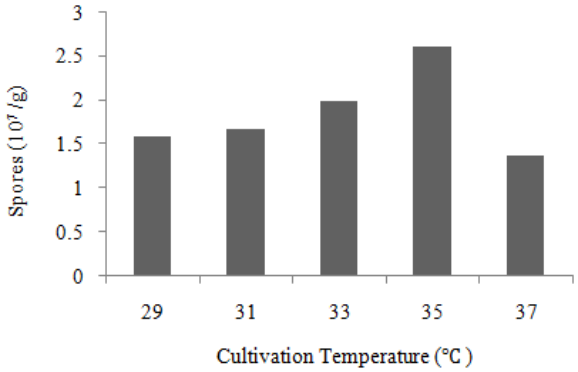

Fig. 8: Effects of cultivation temperature on Monascus mouldstarter sporulation

sufficient carbon source that leading to mycelial growth, sporulation reduced and spores germinated. It's better not to add glucose in Monascus koji preparation.

Effects of cultivation temperature on Monascus sporulation: Figure 8 shows that, Monascus sporulation increased slowly and then decreased as the temperature increased, a maximum of 2.62×10^7/g at temperature 35°C and a minimum number at 37°C. The reason is probably that, when the ambient temperature is high, the temperature in the flask could be higher, inhibiting Monascus growth, thus reducing the number of spores.

Process optimization by orthogonal test of seed koji of monascus: To study the effect of different factors on Monascus sporulation and to confirm the optimum process conditions, this study focused on three main factors (material moisture, incubation time, cultivation temperature) to conduct optimization analysis. Test results were shown in Table 2.

According to direct analysis, range (R) of A was maximal, followed by B and C (R) is minimum. Blank D was not the biggest, indicating the test is was without missing important factors. Thus, order of the factors was: A (moisture content of materials) >B (incubation

Table 3: Results of ANOVA

Item	Quadratic sum (I)	df	Mean square	F	Sig.	Significance
Model	41.818[a]	7	5.974	38.080	0.006	**
Material moisture content	40.761	3	13.587	86.609	0.009	**
Cultivation temperature	0.607	2	0.304	1.935	0.043	*
Incubation time	0.450	2	0.225	1.433	0.411	
Error	0.314	2	0.157			
Total	42.132	9				

(1): p<0.01 stand for highly significant with **; 0.01<p<0.05 stand for significant with *; p>0.05 stand for non-significant; (2): R^2 = 0.993; Adj. R^2 = 0.966

temperature) >C (cultivation time). Trial optimal solution was program A3B3C2, namely the material moisture content was 60%, temperature was 35°C and incubation time was 5d.

ANOVA of orthogonal test: Analysis of Variance (ANOVA) is a very practical and effective method of statistical tests, which can be used to examine the significance of the relevant factors on the test results. In this study, SPSS 19.0 software was used for ANOVA of orthogonal test results. The results were shown in Table 3.

As it can be seen from Table 3, the selected test model was significant, so this model possessed statistical significance. Material moisture content test results were significant, the incubation time test results were significant, while the cultivation temperature test results were non-significant, indicating that, in this test range, the former two had larger influence on the test results and the effect of cultivation temperature on the test results was not obvious.

Verification test: Under optimum conditions, i.e., 0.1 mL inoculation, 5 d incubation time, 10 g filling capacity, 60% material moisture content, 0.2% lactic acid added, 35°C cultivation temperature, to validate the test, the final Monascus sporulation was 3.35×10^7/g, that was higher than the result of orthogonal test, indicating this optimization test was successful.

CONCLUSION

On the basis of single-factor test, we conducted optimization study on the three major factors (material moisture content, incubation time, temperature) that affect the production of purebred Monascus spores through orthogonal trial. Results show that, in the trial-level range, moisture content of materials and cultivation time have larger effect on the test results, while the cultivation temperature has little effect. The optimum conditions in preparation of purebred Monascus koji are: 60% moisture content of materials, 5 d cultivation time, 35°C cultivation temperature, 0.1 mL inoculation, 10 g filling and 0.2% lactic acid. To verify this test scheme, ultimately Monascus sporulation is 3.35×10^7/g, it is higher than all results obtained from orthogonal trial, the orthogonal trial would be then success.

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