CHEMICAL ENGINEERING TRANSACTIONS
VOL. 46, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Peiyu Ren, Yancang Li, Huiping Song

ISBN 978-88-95608-37-2; **ISSN** 2283-9216
DOI: 10.3303/CET1546228

Effects of Culture Conditions on Production of Red Pigment and Citrinin by Fermentation of *Monascus Ruber*

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Effects of carbon source, nitrogen source, and pH value and culture volume on red pigment and citrinin production by fermentation of *Monascus ruber* were investigated in this study. In order to improve the yield of red pigment and to reduce the production of citrinin, the four factors and three levels orthogonal experiment was used in this experiment. Results indicated that carbon source, nitrogen source, pH value and culture volume influenced the production of pigment and citrinin significantly, and the red pigment with highest value was obtained at 30 g/L corn flour, 2 g/L NH_4Cl, pH 4.0 and 100 ml/250ml culture volume, which also without citrinin. The results of the experiments will be helpful to the production of safe pigment, and will improve the usage of pigment in food industry.

1. Introduction

Food color is an important factor related to food sensory quality. However, it usually changes during processing or in storage. The natural food pigments are safety and rich color. The microbial production of natural pigment has a great potential, and *Monascus ruber* has become the main direction in this area, because it is better than the natural pigments from animal and plant sources comparing the price and the stability of the pigment move (Beatriz Kilikian, et al. 2007).

The French scholars Blanc and his colleagues (L. Pastrana et al. 1995) confirmed that some *Monascus ruber* strains can secrete toxic citrinin. The toxicity of citrinin is similar to aflatoxin B and its target is kidney organs. This finding has raised a big question on the safety of natural red pigment and red yeast rice products. Many scholars try to use different methods to control or reduce the *Monascus ruber* citrinin(Yu Huiling, Nie Xiaohua, 2005). However, due to the stability of the red pigment, red pigment color value has been reduced after some processing. Therefore, seeking the kind of high color and low citrinin food colorant becomes the challenge in food industry.

In this paper, a liquid fermentation method is used, through inhibit the generation of citrinin and to improve the color value of red yeast rice. The study concluded that the medium composition and fermentation condition has impact on red yeast *Monascus ruber* generating pigment and citrinin. It determines the medium composition and fermentation conditions of the high-yielding red pigment without producing the citrinin, it provides a theoretical basis for future food security production of red pigment.

2. Materials and Methods

2.1 Strain

Monascus ruber, Separated by the red heart Daqu used by the ShanXi Vinegar.

2.2 Test Equipments

The ZF-III Temperature controlled bio-incubator (by XingTa Agricultural Equipment Factory); TH2-82A model Steam and bath constant temperature oscillator (by Changzhou Guohua Electric Appliance Co., Ltd.); TD4 desktop centrifuge (by Hunan Instrument Plant centrifuge plant); the 22pc visible spectrophotometer (by Beijing Optical Instrument Factory); CS101,-2E Electric Blast Oven (by Chongqing Instrument Co., Ltd.); LC-20 High performance liquid chromatograph (by Shimadzu, Japan)

Please cite this article as: Zhou Z.G., Guo H.Z., Xie C.Y., 2015, Effects of culture conditions on production of red pigment and citrinin by fermentation of monascus ruber, Chemical Engineering Transactions, 46, 1363-1368 DOI:10.3303/CET1546228

2.3 Medium

Slant preservation medium: malt extract agar (g/L): maltose 80, agar 15.

The seed medium (g/L): Maltose 30 g, $NaNO_3$ 2 g, KCL 0.5 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, agar 15 -20 g, distilled water 1000 ml, pH 5.8, 250 mL triangle bottled 100 mL, 21℃ sterilization 20 min, and were inoculated with activation of the good bacteria, and placing a shaking 30℃, 200r/min shaking culture for 3 days.

2.4 *Monascus Ruber* Culture Method (Silvana T. et al. 2008)

2.4.1 Spore Suspension of Production

Sterile water pipette transferred to malt extract agar slant culture slant spores with ring vaccination stripped, dissolved in sterile water, broken up with glass beads so that the spore concentration cells of 5.0×10^6-5.0×10^7 / mL.

2.4.2 *Monascus Ruber* Seed Culture

Flask of 50 mL/250 mL, and inoculated with spore suspension of 5mL, the 200r/min gas bath shaking at 30℃ for 48h.

2.4.3 Shake Flask Fermentation

2.4.3.1 Single factor carbon source fermentation

Fermentation medium (g/L): carbon source, glucose, corn flour, millet flour, maltose, soluble starch as carbon source, pH 7.0, the 3% inoculum size, flask 150 mL, and the rest of the culture conditions with the seed culture medium, shaking culture for 7 days.

2.4.3.2 Fermentation on Each pH Level

Medium composition with the seed culture medium, pH values were 3.5, 4.0, 5.4, 6.0, 7.0 and the remaining culture conditions with a carbon source, single-factor experiment.

2.4.3.3 Fermentation on Each Nitrogen Source Level

Nitrogen sources were used to select sodium nitrate, ammonium chloride, soybean meal, peptone, glutamic acid, and the remaining ingredients with the seed culture medium, the rest of the culture conditions with the carbon source of single-factor test.

2.4.3.4 Fermentation culture on Installed Fluid Volume Level

Medium composition with the seed culture medium, 250 mL flask fluid volume were 75 mL, 100 mL, 125 mL, 150 mL, 175 mL, and the remaining culture conditions with the carbon source of single-factor test,

2.4.3.5 Orthogonal Fermentation

From the experiments of above single factor fermentations, choose top three levels to produce the *Monascus ruber* pigment. Then proceed the four factors and three levels of orthogonal experiment design. The remaining culture conditions are the same as the carbon source, single-factor test.

2.5 Determination of the Indicators and Methods

2.5.1 Color Value (QUAN Guijing et al. 2014)

Water-soluble red pigment: After fermentation, the fermentation broth was filtered with four layers of gauze. Then it was diluted with the amount of distilled water and shaken. Using the distilled water as blank control, measure the OD value of 510 nm and 410 nm with a spectrophotometer.

Alcohol-soluble red pigment: Place the filtered mycelium in 50℃ blast oven and bake for 15-20 min, fully ground. Use ethanol, 75% (volume fraction) of the fermentation broth, shock extraction for 24 h. Centrifugation, diluted with the amount of 75% (volume fraction) ethanol. Using the 75% (volume fraction) ethanol as the blank control, measure the OD value of 510 nm and 410 nm with a spectrophotometer.

Color value = OD × dilution factor

2.5.2 Biomass **(**Wang Hailei, et al. 2011)

Mycelium remained on the upper layers of gauze after the fermentation broth was filtered with four layers of gauze. It was washed with distilled water till the washing liquid colorless. Then the mycelia was transferred to the filter paper dried using the filter paper, weighted which is the wet bacteria weight.

Biomass = weight of wet cells / fermentation liquid volume

2.5.3 Determination on content of citrinin (JyhJye Wang et al., 2004)

After fermentation, mash the fermentation broth and mycelium, imbibe the 10ml of processed fermentation broth, add 20 mL of ethanol, and place them in the oscillation of the water bath for 1h, then 6000r/min centrifuge 10min, and filter through a 0.45 μm membrane for further usage.

Filtrate filter with high performance liquid chromatographic is used to measure the citrinin content. HPLC chromatographic conditions are as follows column temperature: 28℃, mobile phase: (deionized water, phosphoric acid, pH3.0): V (methanol) = 47:53; fluorescence detection: λ ex = 331 nm, λ ex = 500 nm; column: 5μm; Edopse XDB Reversed - phase C. column length and column diameter: 250 mm × 4.6 mm; volume flow rate: 1.0 mL / min; sample volume: the standard sample and the sample are 20μL.

3. Results and Analysis

3.1 Effects of Carbon source on the production of red pigment and citrinin

Choose different carbon sources, the results are shown in Table 1.

Table 1: Effects of carbon source on the production of red pigment and citrinin

Carbon source	Biomass g/ml	Sensory color	Water-soluble U/ml	Alcohol-soluble U/ml	Color value U/ml	Citrinin mg/L
Glucose	0.474	Orange	38.88	100.8	139.68	0.93
Corn flour	2.58	Crimson	129.6	157.68	573.88	0.18
Millet flour	1.46	Rose Red	100.8	136.8	237.6	-
Maltose	1.56	Crimson	108.72	142.56	251.28	1.23
Soluble starch	0.23	Reddish	59.76	64.08	123.84	-

Note: - expressed was not detected.

The results showed that the color value of the red yeast rice is the highest if using corn meal as the sole carbon source, followed by maltose and rice flour as a carbon source. Using glucose and soluble starch as carbon source get the lowest of *Monascus ruber* color. The effect of Carbon sources on the production of citrinin trend is different from the one on red pigment. Using maltose as carbon source, the highest yield of citrinin is 1.23 mg / L, followed by glucose as carbon source. While using the millet flour and soluble starch as the carbon sources, no citrinin is detected.

3.2 Effects of nitrogen source on the production of red pigment and citrinin

Select sodium nitrate, the results are shown in Table 2.

Table 2: Effects of nitrogen sources on production of red pigment and citrinin

Nitrogen source	Biomass g/ml	Sensory color	Water-soluble U/ml	Alcohol-soluble U/ml	Color value U/ml	Citrinin mg/L
$NaNO_3$	1.02	Reddish	24.48	61.2	85.68	-
NH_4Cl	0.58	Red	72.72	48.96	121.68	-
soybean	0.22	Orange	38.16	65.52	103.68	1.05
Peptone	1.32	Yellow	18	34.56	52.56	1.37
Glutamate	0.08	Light yellow	5.76	10.08	15.84	-

Note: - expressed was not detected.

The results showed when ammonium chloride as the nitrogen sources, the amount of mycelia grows the most, and color value of the produced red pigment is the highest. When Soybean as the nitrogen sources, the mycelium grows more than others, and the color value of the produced red pigment is higher than others too. Different type of nitrogen sources can produce quite different red pigment colors. Using soybeans, Peptone and other organic nitrogen as nitrogen source, the fermentation broth is yellow. Using sodium nitrate, ammonium chloride, urea and inorganic nitrogen as the nitrogen source, the fermentation liquid became red. This is very useful for producing different colors of food coloring.

The results showed *Monascus ruber* fails to generate the citrinin if using sodium nitrate, ammonium chloride or glutamate as nitrogen source. It is probably due to the high pH fermentation environment caused by the consumption of the ammonium salt which can significantly raise the pH value and inhibit the metabolism of citrinin formation. Using Soybean powder and peptone as the nitrogen source, citrinin is produced relative high, as 1.05 mg/L and 1.37 mg/L respectively.

3.3 Effects of pH Values on the production of red pigment and citrinin
Choose different pH values, the results are shown in Table 3.

Table 3: Effects of pH values on production of red pigment and citrinin

pH	Biomass g/ml	Sensory color	Water-soluble U/ml	Alcohol-soluble U/ml	Color value U/ml	Citrinin mg/L
3.5	0.13	Light yellow	7.2	17.28	24.48	0.61
4.0	0.2 8	Orange	10.08	16.56	26.64	0.15
5.4	0.68	Reddish	30.24	36	66.24	-
6.0	0.73	Reddish	33.84	37.44	71.28	0.11
7.0	0.50	Light red	25.92	30.96	56.88	-

Note: - expressed was not detected.

As shown in Table 3, the most suitable pH value for forming the *Monascus rubber* is 6.0. The fermentation broth color is red and the color value of the red yeast rice is the highest. If pH value between 5-7, the color value of the red yeast rice is relatively high. If pH value between 3-4, *Monascus ruber* color value is the lowest and the color is light. The reason may be that the pigment was stable and not easily broken down. As for whether fermentation the pH will affect the production of citrinin, the mechanism needs to be further studied.

3.4 Effects of liquid volume on the Production of red pigment and citrinin
In 250 mL flask, use different of the liquid. The results are shown in Table 4.

Table 4: Effects of culture volume on the production red pigment and citrinin

Liquid Volume	Biomass g/ml	Sensory color	Water-soluble U/ml	Alcohol-soluble U/ml	Color value U/ml	Citrinin mg/L
75	0.52	Light yellow	30.24	44.08	74.32	-
100	0.61	Reddish	50.4	54	104.4	-
125	0.41	Light red	40.10	35.05	75.15	-
150	0.21	Reddish	11.12	24.30	35.42	0.12
175	0.08	Pink	18.25	12.25	30.50	-

Note: - expressed not detected.

Monascus ruber has the highest color value when the liquid volume is 100 mL/ 250 mL. It has highest color value for liquid volume 100 mL/ 250 mL, followed by 125 mL/ 250 mL. Since *Monascus ruber* is aerobic microorganisms, liquid fermentation of red yeast is aerobic fermentation with dissolved oxygen. So liquid volume has a significant impact on producing *Monascus ruber* pigment. With the increasing of dissolved oxygen, the production of *Monascus ruber* pigment increase higher and higher. There must be sufficient oxygen supply in order to improve the content of the red pigment. Within the scope of this test, the citrinin content is very low, only a small amount is detected in 150 mL/ 250 mL.

3.5 The Best Ratio Test of Monascus ruber culture conditions

The factor levels are shown in Table 5 and the results of the orthogonal and intuitive analysis are shown in Table 6.

Table 5: Factors and levels

Level	Nitrogen source	Carbon source	pH	Culture volume(ml/250ml)
	A	B	C	D
1	$NaNO_3$	Millet flour	4.0	70
2	NH_4Cl	Maltose	5.4	100
3	Soybean	Corn starch	6	125

Table 6: Results of orthogonal and intuitive analysis

No.	A	B	C	D	Sensory color	Biomass (g/mL)	color value(U/ml)	Citrinin(mg/L)
1	1	1	1	1	Orange	0.04	291.6	0.15
2	1	2	2	2	Pink	2.5	370.0	0.38
3	1	3	3	3	Light red	0.07	298.8	-
4	2	1	2	3	Crimson	2.7	179.2	-
5	2	2	3	1	Purple	1.3	587.8	-
6	2	3	1	2	Crimson	2.9	780.1	0.19
7	3	1	3	2	Orange	0.2	113.4	-
8	3	2	1	3	Purple	0.65	558	0.05
9	3	3	2	1	Pink	1.4	487.8	0. 42
K_1	1057.5	484.2	1629.9	1027.8				
K_2	1107.9	1273.5	1034.1	1360.8				
K_3	1159.2	1566.9	547.2	936				
k_1	352.5	161.4	543.3	342.6				
k_2	369.3	424.5	344.7	453.6				
k_3	386.4	522.3	182.4	312				
R	33.9	360.9	360.9	141.6				

Note: - expressed not detected.

According to R, carbon sources and pH have highest R values, nitrogen source has lowest R value. This indicates that the carbon source and pH have greatest impact on the red pigment, followed by liquid volume, and nitrogen source has little influence. According to the test results, the best *Monascus ruber* culture condition is $A_2B_3C_1D_2$, the 6th in Table 6, the amount of liquid NH_4CL 2 g/L, corn flour 3 0 g/L, pH 4.0, installed liquid volume 100 mL/ 250mL. The results in Table 6 show whether the *Monascus ruber* produces citrinin depends on the culture conditions. In experimental conditions, the content of citrinin was measured as lower

than 0.5 mg/L. It is in line with the relevant provisions of the content of citrinin in the *Monascus ruber* pigment products.

4. Discussions

4.1 Factors affecting the Generation of citrinin

Professor P.J, Blanc states, whether and how much citrinin is produced depends on the amount of Monascus culture methods, culture conditions, strain and media related. The results indicate that the citrinin is not detected in the majority of culture conditions and the detected maximum value of the citrinin is 1.37 mg/L. Patcharee Pattanagul et al. (2008) reported that *Monascus ruber* in potato glucose medium did not produce citrinin, but it produced higher amount of citrinin in the yeast extract medium. Zhou Bo (2009) reported that the ammonium salt has big impact on the metabolism of *Monascus ruber* red pigment and citrinin.

4.2 The Biological Pathways of producing citrinin

Because the citrinin is the secondary metabolites in the late of *Monascus* fermentation production (Li Yun Yan, et al. 2000), many scientists began to study the metabolic pathways of *Monascus* citrinin in recent years. First of all, an acetyl coenzyme A molecule and four malonyl coenzyme A molecules condense pentanone, and then by methylation, condensation, reduction, alkylglycosylation, reduction, oxidation, dehydration and other steps, ultimately producing citrinin. Hassan Hajja (2000) reported on the analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumulation. At present, the metabolic pathways are not yet entirely clear, in particular, one of the enzymes. If this is clarified, it can be regulated according to their metabolic mechanism of *Monascus* fermentation in order to improve the production of red pigment and to reduce the production of citrinin.

Acknowledgements

This work was financially supported by Hebei Province, the development and application of edible and medicinal mushroom resources (YF201411), And by project of Langfang Teachers University (LSZY201304) , and the important discipline of genetics in Hebei Province.

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