

Study of Kojic Acid Extraction Method from Fermentation Broth

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Abstract: Kojic acid was a microbial metabolite produced by many species of *Aspergillus*, *Acetobacter*, and *Penicillium*. Kojic acid was widely used as a food additive for preventing enzymatic browning, and in cosmetics as a skin-lightening or bleaching agent. In this paper, experiment results showed that the fermentation product could be effectively recovered with crystallization processes. Through 3 times crystallization, kojic acid's purity could be able to reach 97.30%, with a total extraction rate above 70%. Activated carbon decolorized the extracts well; the optimum decolorization was done at 70 °C, with 2% of activated carbon added, under natural pH for 30mins.

Key words: Kojic acid extraction, Tropical fruits, Storage and preservation

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I. Introduction

Kojic acid (KA) is an acidic compound produced by fermentation of certain aerobic strains, and can be fermented by using raw materials such as glucose, fructose, sorbose and sugar alcohol. The pure kojic acid is a prismatic or needle-like white crystal, and its chemical name is 5-hydroxy-2-hydroxymethyl-1,4-pyrone. In 1907, Saito Sindo found it in the extract of rice koji. Its molecular structure was determined in 1924. Subsequently, Manabe M et al. conducted a detailed study on kojic acid¹⁻³.

Since the discovery of kojic acid by Japanese scholars, they have been leading the research in this field. Typically, T Futamura et al. of the University of Gifu, Japan, induced NTC to the strain MK107-39 by NTG on the starting strain *A. oryzae* ATCC-22788, and the yield was 7.7 times of that in the original strain⁴. Malaysian scholars R Mohamad and A B. Ariff also conducted a detailed study of *A. flavus* Link 44-1 strain, which finally yielded 45.3 g/L and 33.4 g/L kojic acid using glucose and sucrose, respectively⁵. The kojic acid-producing strain NRRL484 has also been studied by many scholars^{6,7}. Therefore, study of kojic acid extraction method from fermentation broth is significant as part of this area.

II. Materials and Method

2.1 Materials

Strain: *Aspergillusoryzae* M34, preserved in the laboratory.

Purification medium: 3% sucrose, 0.2% NaNO₃, 0.05% KCl, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.01% FeSO₄·7H₂O, 2% agar, 0.01% FeCl₃, 0.01% CaCl₂, pH 7.0, sterilized at 121 °C for 20 min. (Note: FeCl₃ should be sterilized after other ingredients, filtered through a sterile filter and added separately.)

Agarslantculture-medium (PDA medium): 200 g of fresh potatoes, boiled for 1 h, and filtered with two layers of gauze. The filtrate was added with 15 g of glucose, 20 g of agar and filled to volume of 1000 mL, in natural pH, and sterilization at 121 °C for 20 min.

Seed medium: 10% glucose, 0.25% yeast extract, 0.5% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, pH 6.0, sterilized at 121 °C for 20 min.

The basic components of the fermentation medium: 10% tapioca saccharification solution, 0.25% yeast extract, 0.05% MgSO₄·7H₂O, 0.05% K₂HPO₄, 0.001% FeSO₄, and sterilization at 121 °C for 20 min.

2.2 Kojic acid detection

Kojic acid detection

Within a certain range, the absorbance of kojic acid with a certain amount of Fe³⁺ has linear relation with the concentration of kojic acid. The maximum absorption peak was scanned by WFZ800-D3B UV and visible spectrophotometer.

2.3 Extraction methods of kojic acid

The fermentation broth is first filtered by suction to filter out the bacteria in the fermentation broth. The preliminary treated fermentation broth is then subjected to extraction experiments of different methods.

(1) Direct concentration crystallization

300 mL of the initially treated fermentation broth was concentrated in a rotary evaporator at 70 °C to a small amount of crystals, which was poured out, washed with a small amount of water, and the solution was combined. Crystallize at 4°C for 24 h (stirred several times in the middle). The first crystal was collected by vacuum filtration, dried under vacuum, and weighed. The crystals were redissolved in distilled water, concentrated, crystallized, and the second crystal was collected, vacuum dried, and weighed. The same operation gave the third crystal. The purity of the product was analyzed and the extraction rate was calculated. The mother liquor obtained by the two crystallizations was concentrated and crystallized in the same operation as above, and the purity and extraction rate of the product were calculated in the same manner.

(2) Extraction method

In order to determine the effect of different amounts of ethyl acetate on the yield of kojic acid extraction, 300 mL of fermentation broth (containing 12.45 g of kojic acid) was added to ethyl acetate for extraction according to the fermentation broth: ethyl acetate (volume ratio) = 1:0.8, 1:1.0, 1:1.2, 1:1.4. After extracting with ethyl acetate, the mixture was shaken for 1 min every 2 min and repeated for 10 times, and then allowed to stand for 1 h. The ethyl acetate layer was separated, and subjected to a rotary evaporator to recover ethyl acetate. The obtained product was vacuum dried, weighed, and analyzed to determine the purity of the product, and the extraction rate was calculated.

(3) Precipitation method

Take 300 mL of fermentation broth (containing 12.45 g of kojic acid), adjust to pH 6.5 with NaOH, and then added a slight excess of ZnSO₄•H₂O (calculated by adding 7.8 g and actually adding 8.5 g). Stir well, vacuum filter, collect zinc kojic acid precipitation, dry, and weigh. Add 98% of the theoretical value of H₂SO₄ (8.8 mL, added slightly after dilution). The acid solution was heated at 50°C for 0.5 h, and then the acid hydrolyzate was directly concentrated until crystal formation. Crystallization at 4°C for 24 h (stirring in the middle), vacuum filtration, collection of crystals, vacuum drying, weighing. Analyze the product purity and calculate the extraction rate.

2.4 Decolorization of activated carbon

Effect of the amount of activated carbon on the yield and decolorization of the product: 100 mL of the filtered kojic acid fermentation broth was taken in each beaker from No. 1 to No. 5, and 1.0 g, 2.0 g, 4.0 g, 6.0 g, and 8.0 g of activated carbon were added respectively; the mixture were decolorized at a natural pH for 30 min under 50°C, and then filtered. Acid content, clarity, kojic acid yield and decolorization rate were detected.

(1) Effect of decolorization time on product yield and decolorization effect

10, 20, 30, 40, 50 min were used as time gradients respectively. 2% activated carbon was added at 50 °C, and the pH was natural. The filtrate was used to determine the content of kojic acid, clarity, yield of kojic acid and decolorization rate.

(2) Effect of pH on product yield and decolorization

pH 2, 3, 4, 5, and 6 were used as gradients respectively. 2% activated carbon was added at 50 °C for 30 min. The filtrate was measured for kojic acid content, clarity, kojic acid yield and decolorization rate.

(3) Effect of temperature on product yield and decolorization effect

40, 50, 60, 70, 80 °C were used as time gradients, 2% activated carbon was added, the pH was natural, and the color was decolorized for 30 min. The filtrate was measured for kojic acid content, clarity, kojic acid yield and decolorization rate.

III. Results and analysis

3.1 Effect of different extraction methods on kojic acid purity

(1) Extraction of kojic acid by concentrated crystallization

300 mL of the fermentation broth (containing 12.45 g of kojic acid) was concentrated in a vacuum in a rotary evaporator, and the first crystallization was carried out, the crystals were collected, dried under vacuum, and m₁ (9.41 g) was weighed. The crystals were redissolved in distilled water, concentrated, crystallized, and the second crystal was collected, dried under vacuum, and weighed m₂ (7.81 g). The same operation gave m₃ (6.62 g). The purity of the product was analyzed and the extraction rate was calculated. The mother liquor obtained by the two crystallizations was concentrated in the same manner as above, and crystallized at a low temperature. The purity and extraction rate of the product from three times crystallization were shown in Table 1.

Table 1 Extracting KA by crystallization

Samples	1 st crystallization	2 nd crystallization	3 rd crystallization	1 st mother liquid	2 nd mother liquid
Purity	68.30%	95.80%	97.30%	63.90%	60.41%
Extraction rate	75.60%	83.00%	84.80%	71.80%	41.40%

As can be seen from Table 1, as the number of extractions increases, the purity of the product continues to increase. Through three crystallizations, the purity of the product can reach 97.30%. The extraction rate is also getting higher, and the color of the extract is getting lighter. However, in the treatment of the mother liquor, it was found that the extraction rate of the product was significantly low, from 71.80% of the primary mother liquor to 41.40% of the secondary mother liquor, and the purity of the product decreased slightly. The reason is that the concentration of the secondary mother liquor is low, and the loss is more in the process of forming crystallization. It is calculated that the extraction rate of kojic acid is 53.2% by three times of crystallization, and if the mother liquor is not crystallized, the extraction rate is 69.29%.

(2) Extraction of kojic acid by ethyl acetate

Different ratios of fermentation both with ethyl acetate (1:0.8; 1:1.0; 1: 1.2; 1:1.4) were tested to determine the effect of ethyl acetate on the extraction yield of kojic acid, and the results were shown in Table 2.

Table 2 Extracting KA by ethyl acetate

Fermented broth: ethyl acetate (v/v)	1:0.8	1:1.0	1:1.2	1:1.4
Final product mass (g)	emulsified	2.34	2.72	3.17
Purity (%)	—	97.20	99.12	99.54
Extraction rate (%)	—	18.80	21.85	25.46

As can be seen from the table, higher doses of ethyl acetate facilitate extraction and the product purity is very high. But the extraction rate is not acceptable. The extraction rate of about 20% is quite low compared with other extraction methods.

(3) Precipitation method for extracting kojic acid

8.0g of ZnSO₄•H₂O was added and sufficiently reacted with kojic acid to form zinc kojate. The experiment results are shown in Table 3.

Table 3 Precipitation method extracts KA

NO.	1	2	3	Average
Zinc kojate (g)	11.32	10.9	11.25	11.16
KA (g)	10.26	9.51	9.83	9.87
KA purity (%)	97.41	98.18	98.31	97.97
Extraction rate (%)	72.41	66.39	68.96	69.25

From the results, we can see the precipitation method can ideally extract the corresponding products, and the average extraction rate is about 70%. Although the purity of the product is not as good as the extraction method, it can reach 98%, which can basically meet the production needs. However, the ZnSO₄ precipitation method is cumbersome and consumes the raw material ZnSO₄. It is not economical compared to the direct crystallization method, and therefore is not an ideal extraction method as well.

In summary, the above extraction methods can obtain more than 97% of high-purity kojic acid. There is a certain difference in the extraction rate, the extraction rate of ethyl acetate is the lowest, and only about 20%; the crystallization method and the precipitation method are about 70%. Although the above methods can obtain kojic acid with higher purity, the cumbersome operation steps are quite different except for the difference in extraction rate. Among them, the concentrated crystallization method is the simplest operation, and has great advantages compared with the other two methods. The extraction method and the precipitation method are not only difficult to operate, but also require additional reagents such as ethyl acetate and ZnSO₄, which significantly increases the production cost. Therefore, considering the comprehensive consideration, the concentrated crystallization method is the most ideal extraction method for kojic acid.

3.2 Decolorization of activated carbon

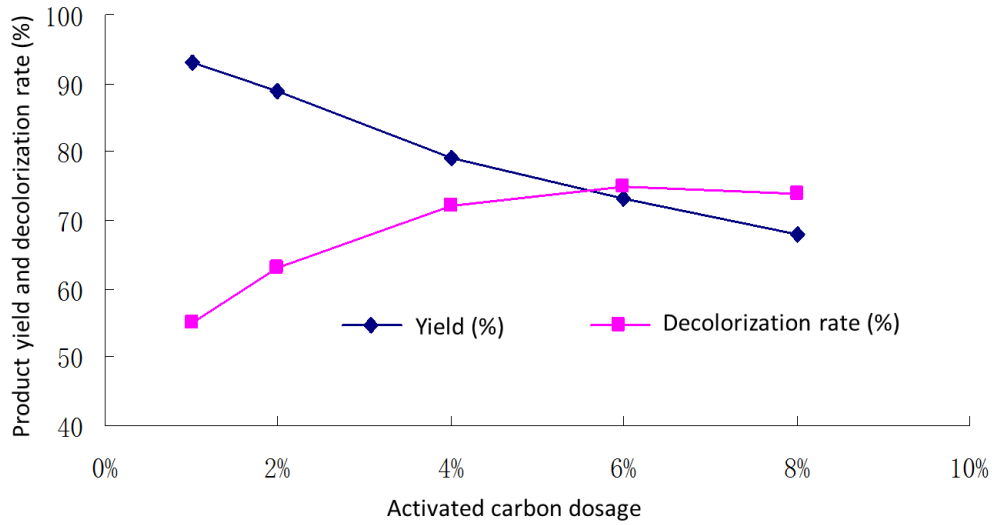


Figure1. The influence of decoloring amount of active carbon on yield of KA and color removal effect

In the Figure1, the larger the amount of activated carbon additive, the better of decolorization effect, but the yield of the product is reduced. This shows that activated carbon adsorbs some products. Therefore, the experiment must choose a suitable dose, which can not only ensure the decolorization effect, but also ensure a certain product yield. For comprehensive consideration, choose 2% as the best dose to use.

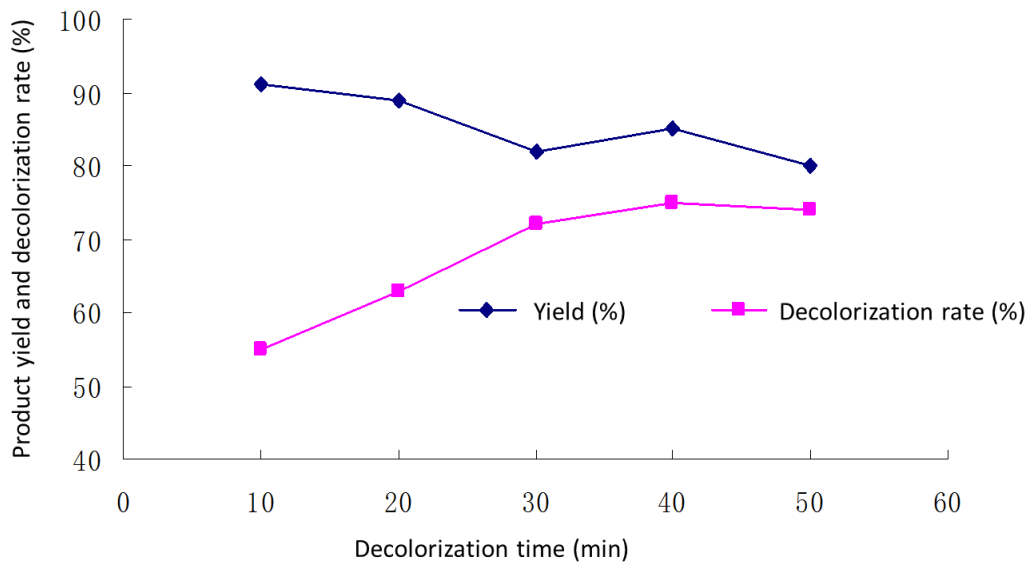


Figure 2. The influence of decoloring time on yield of KA and color removal effect

The decolorization time is a key factor for the full contact of the activated carbon particles with the adsorbed material. It can be seen from Figure 2 that during the decolorization time of 30 min, the decolorization rate increases with the prolongation of decolorization time, and tends to be stable after 30 min. Therefore, the decolorization time of 30 min can basically make the product decolorize to be stable. Under these conditions, the product yield will not be greatly affected.

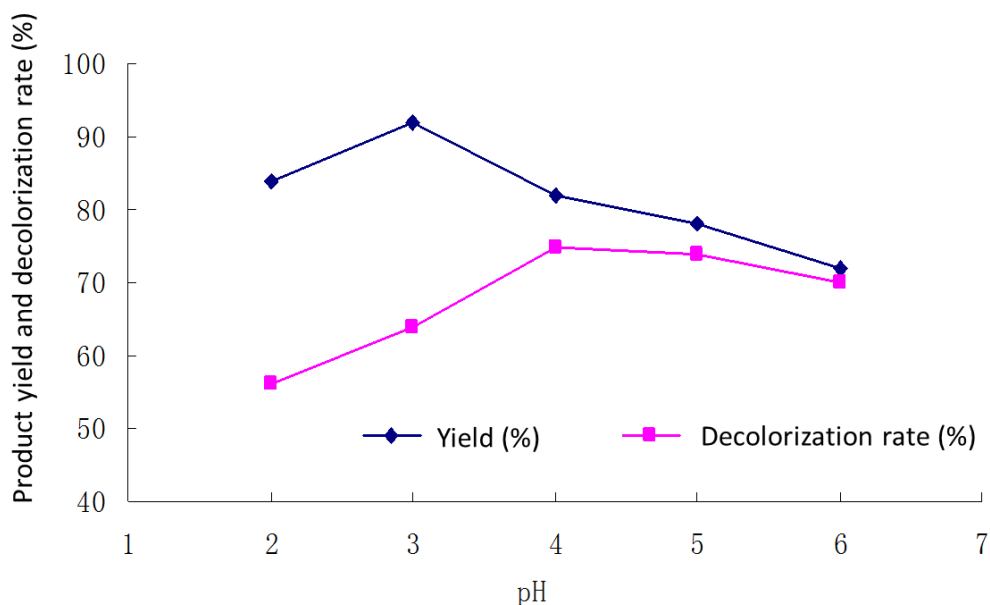


Figure 3. The influence of decoloring pH on yield of KA and color removal effect

The natural pH of the activated carbon used in the experiment is generally about 3 to 4. As can be seen from Figure 3, when the pH is between 3 and 4, the decolorization effect and product yield of the product can be well maintained. Therefore, it is sufficient to maintain the pH during the decolorization process without artificial control.

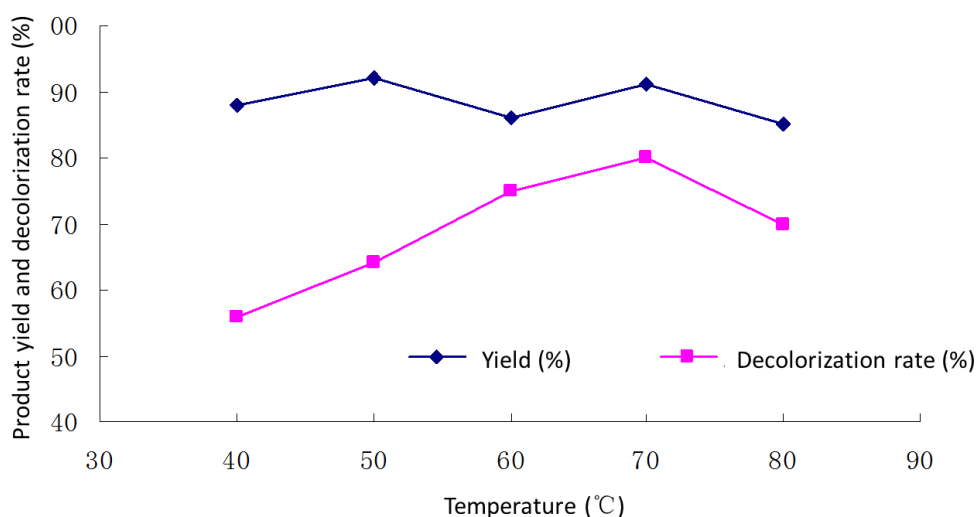


Figure 4. The influence of decoloring temperature on yield of KA and color removal effect

As can be seen from Figure 4, the 70 °C decolorization rate reached the highest value. Under the condition of high temperature, activated carbon can promote adsorption due to the acceleration of molecular motion, thereby improving the decolorization effect. However, the adsorption of activated carbon is accelerated at a high temperature, and the detachment speed is also accelerated. Therefore, the decolorization effect does not increase with an increase in temperature, but occurs at an optimum state at 70 °C. Observing the yield of the product, it was found that it was not affected by the temperature. Therefore, 70 °C was selected as the maintenance temperature during decolorization.

IV. Summary

In this paper, the effects of three extraction methods on the extraction rate of kojic acid were studied. Compared with the extraction method and the precipitation method, the crystallization method can extract kojic acid more easily and efficiently. Through 3 times of crystallization, the purity of the product can reach 97.30%, and the total extraction rate is about 70%. This paper determines the process conditions for the decolorization of activated carbon. The experimental results show that the decolorization effect of activated carbon is better. The best use condition is: adding 2% activated carbon at 70 °C, decolorizing in natural pH for 30 min.

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