Effect of culture conditions and medium compositions on kojic acid production by *Aspergillus oryzae* ATCC 10124

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**Abstract.** Kojic acid is a secondary metabolite produced by some strains of *Aspergillus* spp and has been exploited commercially in food and cosmetic products. The objective of this work was to optimize the cultivation conditions of *Aspergillus oryzae* ATCC 10124 prior to triggering the fermentation process using organic broken rice noodles (OBRN) as a carbon source. Trials with various carbon and nitrogen sources show that 10% glucose and 0.05% yeast extract with ammonium sulfate was favorable for *A. oryzae* to produce kojic acid (1.58 g/L at day 4 of cultivation). Initial pH and agitation rate significantly affected the kojic acid formation. The maximum quantities of kojic acid were obtained when the pH of the medium was at 2.5, and shaking was at 200 rpm, at 1.60 g/L and 1.65 g/L, respectively. When the carbon source was changed to OBRN, the substrate was saccharified by alpha-amylase and glucoamylase, and 100% of the hydrolysate was used to replace glucose for the kojic acid fermentation. Kojic acid content increased when using OBRN, at 1.52 g/L, and was comparable to that yielded from the fermentation with glucose (1.58 g/L).

1. Introduction
Kojic acid was first isolated by Satio (1907) [1] from the mycelium of *Aspergillus oryzae* on steamed rice. The chemical structure of kojic acid was identified as 5-hydroxy-2-(hydroxymethyl)pyran-4-one [2]. Kojic acid is a secondary metabolite produced by different types of fungi utilizing various sub-
strates during aerobic fermentation. It has many potential industrial applications in the food, agriculture, cosmetics, and pharmaceutical industries [3]. For instance, kojic acid can be used in preserving agricultural products, particularly vegetables and fruits, during storage by inhibiting the action of polyphenol oxidase (PPO). It can suppress hyperpigmentation in human skin and restrains the formation of melanin in human skin by inhibiting the tyrosinase enzyme that is responsible for skin pigmentation [3].

Kojic acid was produced from various carbon sources as the secondary metabolite synthesized via phosphorylated intermediates, as reported by Kahn et al. (1997) [4] and medium composition is one of the crucial factors for high kojic acid production. Several monosaccharides have been investigated; however, glucose was reported to generate the highest yield of kojic acid in A. oryzae (24.2 g/L) [5] and A. flavus (39.9 g/L) [6], when 100 g/L of glucose was supplied. The type of nitrogen sources influenced both fungus growth and kojic acid production. Organic nitrogen sources were preferable to inorganic nitrogen sources, as was shown in the work of Rosfrizan and Ariff (2000) [6] in which 5 g/L yeast extract resulted in the maximum kojic acid concentration (39.9 g/L).

A previous study showed that the optimal pH for kojic acid production by a different strain of Aspergillus oryzae ranged between 3 to 5. Lui et al. (2016) [7] reported the highest kojic acid production in the medium with pH 3 (35.27 g/L) by Aspergillus oryzae BCRC 30010. Optimization of oxygen provision could enhance the effectiveness of glucose catabolism by the aerobic microorganism in the early stage of fermentation. Consequently, the intermediates obtained during the growth phase are essential for the biosynthesis of kojic acid. Examples of aeration and agitation on kojic acid production were reported by Ariff et al. (1996) [8]. They stated that in A. flavus, the production of kojic acid decreased significantly when the dissolved oxygen tension levels during the initial phase of growth were lower than 80% [8].

There are many advantages to using microorganisms for the production of natural products; for example, microbial products can be produced by large-scale fermentation; the fermentation process is not subject to seasonal variation or climatic factors; and new cultivation techniques and microbial strain selection will help to improve the productivity. Production costs can be minimized by using cheap starting materials or industrial by-products. Several fungi, such as Aspergillus awamori, Acremonium nigricans, and Aspergillus oryzae, were used for 1-(3,4-dihydro-2H-pyrrol-5-yl) ethanone (2-acetyl-1-pyrroline) formation (a major flavour compound in aromatic rice) [9]. In addition, Aspergillus oryzae has been reported as being of low pathogenic potential, and does not produce aflatoxins or any other carcinogenic metabolites; therefore, it is an excellent vehicle for the safe production of low-risk products. Spores of Aspergillus oryzae NRRL 484 (ATCC 10124) were entrapped in calcium alginate gel beads for the production of kojic acid [10]. The immobilized cells in flask cultures were accumulated with a concentration as high as 83 g/L. Liu et al. (2016) [7] reported that high kojic acid production (83.47 g/L) could be obtained in a PCS-immobilized bioreactor by Aspergillus oryzae BCRC 30010. However, all kojic acid formation in all reports mentioned above was measured by spectrophotometry, which depends on the color reaction that may be subject to interference from other ingredients in the medium.

Organic broken rice noodles (OBRN) are a by-product of organic rice noodle processing. The cost of organic rice is high compared to normal rice used in noodle production, but this by-product is sold cheaply as animal feed ingredients. The OBRN are good sources of carbon, as they consist of 86.65% carbohydrate, which can be used for kojic acid production. Enzymatic hydrolysis could be applied to digest polysaccharide into fermentable sugars, as well as offering the advantages of higher conversion, minimal by-product formation, low energy requirements, and mild operating conditions, compared to other chemical conversions [11]. Consequently, the objective of this research was to study the effect...
of the culture conditions and medium compositions, including carbon sources and nitrogen sources, on the formation of kojic acid. The potential use of organic broken rice noodles as the carbon source for fermentation was also investigated.

2. Material and methods

2.1. Microorganism and spore preparation

Aspergillus oryzae ATCC 10124 was purchased from American Culture Collection Center, USA. The liquid spores were prepared by cultivation on PDA for 4-7 days and harvested from PDA agar plates with 1% (v/v) of tween solution. The spore suspension was counted using a counting chamber to adjust spore suspension to around 10^6 spores/mL. Kojic acid was obtained from Alfa Aesar (USA) and all chemicals used were of analytical grade.

2.2. Medium and culture conditions for kojic acid production

A base medium with the following composition was used: 100 g/L of each carbon source, 0.5 g/L of each nitrogen source, 0.5 g/L KH₂PO₄, 0.5 g/L K₂HPO₄, 0.5 g/L MgSO₄.7H₂O, and 1 ml/L of MnSO₄·H₂O, FeSO₄·7H₂O, and ZnSO₄·7H₂O [10]. To investigate the effect of the different carbon sources (glucose, sucrose, and fructose) and nitrogen sources (yeast extract, peptone, ammonium sulfate, yeast extract with ammonium sulfate, and peptone with ammonium sulfate), different sugars and nitrogen sources were substituted. The effect of medium pH was investigated at pH levels of 2, 2.5, and 3. All submerged fermentations were carried out with 50 ml of medium in a 250 ml Erlenmeyer flask, and incubated at 30 °C and shaken at 150, 200 and 250 rpm on a shaking incubator (LabTech, Korea). OBRN were hydrolyzed using alpha-amylase and glucoamylase prior to being used as a carbon source for kojic acid production.

2.3. Analytical method

Three ml of mold suspension was taken every day from the culture media described above. The sample was filtered using Whatman paper No. 1 while the residues were dried in a hot-air oven at 80 °C overnight in order to measure the cell dry weight. The residual sugar in the filtrate was analyzed using the modified dinitrosalicylic acid (DNS) method [12]. The pH of the culture suspension was measured using a pH meter. The filtrate was again filtered through a 0.45 micron paper filter and degassed before injection to high-performance liquid chromatography (HPLC) to analyse the kojic acid content in the culture broth with a C18 column, 4.6x150 mm (Luma 5 um), and a UV detector at 270 nm. The mobile phase was carried out with a mixture of 1% acetic acid (solvent A, in MilliQ water) and acetonitrile (solvent B). The elution was performed from 0 to 10 min using isocratic 5% solvent B, 10.1-14 min, isocratic 60% solvent B, and a re-equilibration period of 6 min with 5% solvent B used between individual runs. A standard curve for kojic acid was prepared for the calculation. In addition, kojic acid was measured in parallel by the colorimetric method [13]. One ml of cultured medium was added to 4 ml of 1% ferric chloride, and absorbance was read at 540 nm by a spectrophotometer.

2.4. Statistical analysis

All experiments were performed using one-way analysis of variance, and mean comparison was tested using Duncan’s multiple range test for the differences between mean at p<0.05.
3. Results and discussion

3.1. Effect of carbon and nitrogen sources on kojic acid production

Figure 1 shows the HPLC chromatogram of the standard kojic acid, which indicates a retention time of 4.247 min. For the kojic fermentation, significant differences in kojic acid formation with different carbon and nitrogen sources are shown in Table 1. It was clear that when glucose was a carbon source, the kojic acid concentration was significantly higher than with the other C-sources. The amount of kojic acid was enhanced when yeast extract and (NH₄)₂SO₄ were added in equal amounts (1.58 g/L at day 4). Glucose can be directly converted to kojic acid through multi-step enzyme reactions [6]. Fructose and sucrose in this work contributed to lowering the kojic acid concentration significantly. This was in agreement with the work of Kitada et al. (1967) [5] and Rofarizan and Ariff [6]. Sucrose is a disaccharide, which must be hydrolyzed to glucose and fructose by activating an invertase enzyme secreted by the fungus used in the fermentation. The C₆ precursor derived from glucose, which was in the form of pyranose, was more favourable to conversion directly to kojic acid than the furanose form of fructose [5, 6].

The medium with fructose as the carbon source resulted in a very low kojic acid concentration (0.02 g/L). The selection of nitrogen sources for the fermentation medium is important for kojic acid fermentation. Yeast extract and peptone contain vitamins, minerals, and oligoelements for kojic acid production [14-15]. On the other hand, Kwak and Rhee (1992) [10] reported that conidia of Aspergillus oryzae NRRL 484 immobilized in Ca-alginate beads gave a high level of kojic acid, up to 83 g/L (measured by a spectrophotometer), when the fungus was cultivated using 0.5 g/L yeast extract and 0.75 g/L (NH₄)₂SO₄ as nitrogen sources. In the work of Liu et al. (2016) [7] Aspergillus oryzae BCRC 30010 was immobilized in a plastic composite support (PCS) bioreactor with a nitrogen-deficient medium for kojic acid production. The concentration of immobilized culture for kojic acid production was 83.47 g/L after three cycles of cultivation [7]. Several studies have reported high content of kojic acid production analyzed by spectrophotometry and a few reports covered the determination of kojic acid using HPLC [16].

The measurement of kojic acid in our experiment using HPLC was 1.5 g/L, while it was calculated at 30.19 g/L when the same sample was analyzed by the spectrophotometry method. Though spectrophotometry is widely accepted as a useful tool in quantifying many organic substances, when being used with multi-components, particularly the culture media, higher absorbance may occur. Hence, we preferred to express the concentration of kojic acid derived from HPLC measurement rather than that shown by the spectrometer.
Table 1 Kojic acid production influenced by different carbon and nitrogen sources.

| Carbon sources | Nitrogen sources | Kojic acid content (g/L) |
|----------------|------------------|--------------------------|
| (100 g/L)      | (0.5 g/L)        |                          |
| Glucose        | Yeast extract    | 1.13\(\pm\)3.54\(^c\)   |
|                | Peptone           | 0.92\(\pm\)5.66\(^e\)   |
|                | \((\text{NH}_4)_2\text{SO}_4\) | 1.14\(\pm\)14.14\(^b\) |
|                | Yeast extract+\((\text{NH}_4)_2\text{SO}_4\) | 1.58\(\pm\)1.07\(^a\) |
|                | Peptone+\((\text{NH}_4)_2\text{SO}_4\) | 1.03\(\pm\)7.07\(^d\) |
| Sucrose        | Yeast extract    | 0.21\(\pm\)8.49\(^f\)   |
|                | Peptone           | 0.10\(\pm\)1.41\(^i\)   |
|                | \((\text{NH}_4)_2\text{SO}_4\) | 0.15\(\pm\)4.24\(^{hi}\) |
|                | Yeast extract+\((\text{NH}_4)_2\text{SO}_4\) | 0.16\(\pm\)2.12\(^g\) |
|                | Peptone+\((\text{NH}_4)_2\text{SO}_4\) | 0.14\(\pm\)5.67\(^i\) |
| Fructose       | Yeast extract    | 0.02\(\pm\)0.02\(^m\)   |
|                | Peptone           | 0.06\(\pm\)0.06\(^k\)   |
|                | \((\text{NH}_4)_2\text{SO}_4\) | 0.02\(\pm\)0.02\(^m\) |
|                | Yeast extract+\((\text{NH}_4)_2\text{SO}_4\) | 0.03\(\pm\)0.03\(^m\) |
|                | Peptone+\((\text{NH}_4)_2\text{SO}_4\) | 0.05\(\pm\)0.02\(^l\) |

Values are mean \(\pm\) SD of triplicate samples
\(^a\text{--}m\) = the different letters within a column for each attribute constituent are significantly different (p<0.05).

The quantity of kojic acid increased gradually from the first days to a maximum value (1.58 g/L), along with a decrease in the reducing sugar concentration, while pH did not change much during the fermentation (Figure 2). The fungus growth was highest at day 2 (5.95 g/L). The kojic acid fermentation was categorized as a non-growth associated route, where the fermentation comprised both a growth phase and a production phase [15]. However, some kojic acid accumulated in the culture may have been consumed in order to produce other organic acids, after all supplies of glucose have been utilized [17]. Hence, the occurrence of kojic acid reduction appears [18].
Figure 2. Time course of kojic acid production on pH, cell dry weight, kojic acid content, and reducing sugar from the fermentation medium with glucose, yeast extract, and ammonium sulfate and cultivated at 30 °C, 200 rpm.

3.2. Effect of pH and the agitation rate on kojic acid production

Generally in fungi, secondary metabolites are produced in the late log and stationary phases, in which the culture condition has already been acidified by several primary metabolites. In addition, the quantity of kojic acid increased while glucose concentration decreased linearly with incubation time. The results of kojic acid production at different pH levels and agitation rates are shown in Figure 3. The highest amount of kojic acid appeared when the mold had been grown in a pH 2.5 medium (1.62 g/L) at day 4 of the cultivation. The results were in accordance with the study by Rosfarizan et al. (2000) [6] who reported that the optimal pH for the kojic acid producing enzyme was around pH 3.5, during which the pH stress may affect the production of secondary metabolism in both Aspergillus oryzae and Aspergillus flavus [19]. In addition, the secondary metabolites are formed in the late log and stationary phases, during which the fermentation has already been acidified by primary metabolites [20].

The oxygen requirement is one of the approaches to maximize kojic acid production. The results in Figure 2 (d-f) indicate that the oxygen requirement at 200 rpm significantly yielded higher kojic acid quantities (1.65 g/L at day 4) than those at 150 and 250 rpm. However, there were no significant differences in the mycelia dry weight. The secondary metabolites and enzymes produced by various fungal species are subject to the culture conditions, temperature and pH of the medium, composition of the substrate, types of inducers, and inoculum used during the experiments [21]. This was in agreement with this work in order to find the optimum conditions required for A. oryzae to produce kojic acids.
Figure 3. Time course of kojic acid production on pH, cell dry weight, kojic acid concentration and reducing sugar (a: pH 2, b: pH2.5, and c: pH 3) and agitation rate (d: 150 rpm, e:200 rpm, and f:250 rpm) cultivated at 30 °C.

3.3. Use of OBRN as a carbon source in kojic acid fermentation
In order to investigate the potential use of OBRN as a carbon source (instead of glucose), OBRN were hydrolysed in the optimum conditions obtained from earlier experiments. OBRN were hydrolyzed using 1 % alpha-amylase at pH 6.5 in a water bath controlled at 85º C, and shaken at 100 rpm for 1 h. Further reaction was carried out with 0.5% glucoamylase at pH 4.5, in a water bath at 65º C, and shaken at 100 rpm for 21 h. The kojic acid produced, as well as the reducing sugar consumption and growth of *A. oryzae* ATCC 10124 for up to 8 days of incubation, are displayed in Figure 4.

Figure 4. Time course of kojic acid production on pH, cell dry weight, kojic acid content, and reducing sugar, in fermentation using OBRN cultivated at 30 °C, 200 rpm.

The fungus growth was rapid during the initial stage of the fermentation and decreased from day 4, while the highest amount of kojic acid produced was 1.52 g/L at day 7 of the cultivation. Kojic acid was the secondary metabolite found after the rapid growth of the fungi. Rostarizan *et al.* (1998) [22] reported kojic acid production at 23.5 g/L (measured by spectrophotometer) when 100 g/L of sago starch was used from *Aspergillus flavus* strain S33-2. The production of kojic acid in our experiment was not as high, since we express the measurement of kojic acid by HPLC. However, when the same sample was read by a spectrophotometer, it was much higher, at about 30 g/L.

4. Conclusion
Kojic acid fermentation by *Aspergillus oryzae* ATCC 10124 was optimized when cultured in 100 g/L of glucose and 0.5 g/L of yeast extract with ammonium sulfate at an initial pH of 2.5 and incubated at 30 °C and 200 rpm shaking. The highest kojic acid concentration obtained was 1.61 g/L. When the organic broken rice noodles were hydrolyzed and used as the carbon source, 1.52 g/L of kojic acid was
obtained at day 7 of the fermentation. The results show the possibility of using organic broken rice noodles as a carbon substrate for kojic acid production.

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**References**

[1] Saito K 1907 Über die Säurebinding von *Aspergillus oryzae*. *The Botanical Magazine Tokyo* 21 7–11.
[2] Yabuta T 1924 The constitution of kojic acid, a δ-pyrene derivative formed by *Aspergillus flavus* from carbohydrates. *Chem. Soc.* 125 575-587.
[3] Saeedi M, Eslamifar M and Khezri K 2019 Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomed. Pharmacother.* 110 582-93.
[4] Kahn V, Lindner P and Zakin V 1997 Effect of kojic acid on the oxidation of N-acetyldopamine by mushroom tyrosinase. *J. Agric. Food Chem.* 45 (11) 4460-65.
[5] Kitada M, Ueyama H, and Fukimbara T 1967 Studies on kojic acid fermentation (I) cultural condition in submerged culture. *Ferment. Technol.* 45 1101-07.
[6] Rofarizan M and Ariff AB 2000 Kinetics of kojic acid fermentation by *Aspergillus flavus* using different types and concentrations of carbon and nitrogen sources. *J. Indus. Microbiol. Biotech.* 25 20-4.
[7] Lui J M, Yu T C, Lin S P, Hsu R J, Hsu K D and Cheng K C 2016 Evaluation of kojic acid production in a repeated-batch PCS biofilm reactor. *J. Biotechnol.* 218 41-8.
[8] Ariff A B, Salleh M S, Ghani B, Hassan M A, Rusul G and Karim MIA 1996 Aeration and yeast extract requirements for kojic acid production by *Aspergillus flavus*. *Enzyme Microb. Technol.* 19 545-50.
[9] Rungsardthong V and Noomhorm A 2005 Production of 2-acetyl-1-pyrroline by microbial cultures. *Flavour Fragr. J.* 20 710-14.
[10] Kwak M and Rhee J S 1992 Cultivation characteristics of immobilized *Aspergillus oryzae* for kojic acid production. *Biotechnol. Bioeng.* 39(8) 903-6.
[11] Amnuaycheewa P, Rodiahwati W, Sanvarinda P, Cheenkachorn K, Tawai A and Sriariyanun M 2017 Effect of organic acid pretreatment on Napier grass (*Pennisetum purpureum*) straw biomass conversion. *KMUTNB Int. J. App. Sci. Technol.* 10(2) 107-17.
[12] Miller G L 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31(3) 426-28.
[13] Bentley R 1957 Preparation and analysis of kojic acid. *Methods Enzymol.* 24 238-41.
[14] Lin M T, Mahajan J R, Dianese J C and Takatsu A 1976 High production of kojic acid crystals by *Aspergillus parasiticus* UNBF A12 in liquid medium. *Appl. Environ. Microbiol.* 32 298-9.
[15] Chaudhary J, Pathak A N and Lakhawat S 2014 Production technology and applications of kojic acid. *Annu. Res. Rev. Biol.* 4(21) 3165-96.
[16] Masse M O, Duvallet V, Borremans M and Goeyens L 2001 Internat. J. Cosmetic Sci. 23 219-232.
[17] Clevström, G and Ljunggren H 1985 Aflatoxin formation and the dual phenomenon in *Aspergillus flavus* Link. *Mycopathol.* 92 129-39.
[18] Durga Devi K B, Vijayalakshmi P, Shilpa V and Kumar BV 2015 Optimization of cultural
parameters for cost effective production of kojic acid by fungal species isolated from soil. *MRJI.* 7(5) 255-68.

[19] Rosfarizan M, Ariff A B, Hassan M A and Karim M I A 2000 Influence of kojic acid on kojic acid fermentation by *Aspergillus flavus.* Pakistan J. Biol. Sci. 3(6) 977-82.

[20] Shwab E K., Keller N P 2008 Regulation of secondary metabolite production in filamentous ascomycetes. Mycological Res 112(2) 225-230.

[21] Isanapong J, Kraloeakpaiboon T and Noiniyom W 2017 Utilization of organic wastes for Laccase production by *Pleurotus ostreatus.* KMUTNB Int. J. Appl. Sci. Technol. 10(2) 239-44.

[22] Rosfarizan M, Ariff A B, Hassan M A and Karim M I A 1998 Kojic acid production by *Aspergillus flavus* using gelatinized and hydrolyzed sago starch as carbon sources. *Folia Microbio. (Praha)* 43(5) 459-64.