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Kojic acid production from rice by *Amylomyces rouxii* TISTR 3182 and *Aspergillus oryzae* TISTR 3259 and its cosmeceutical potential

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ABSTRACT: This study aimed to investigate kojic acid production from the fermentation of rice by using *Amylomyces rouxii* TISTR 3182 and *Aspergillus oryzae* TISTR 3259. Steamed rice was fermented by *A. rouxii* followed by *A. oryzae* for 15 days, and mixed and separated cultures of both strains were compared. Kojic acid was analysed using Bentley's colorimetric method while glucose was analysed by the dinitrosalicylic acid (DNS) method. The results showed that separation of *A. rouxii* from rice medium before the addition of *A. oryzae* produced more kojic acid than the mixed culture, reaching a maximum yield of approximately 1.54 ± 0.10 g/l at day 10, pH 4 and 27 ± 2 °C. The fermented broth was evaluated for antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, producing a maximum inhibition of 84 ± 11% and antityrosinase activity by dopachrome method with a maximum inhibition of 89 ± 11%. It may be concluded that microbial fermentation of rice could provide a good source of kojic acid. Separation of *A. rouxii* TISTR 3182 before the addition of *A. oryzae* TISTR 3259 in rice medium resulted in more effective kojic acid production. The fermented mixtures may have the potential to be further utilized as functional food and cosmetic ingredients, providing antioxidant and whitening activity.

KEYWORDS: *Amylomyces rouxii* TISTR 3182, *Aspergillus oryzae* TISTR 3259, kojic acid, rice, antioxidant, antityrosinase activity

INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone) is an organic acid that has various industrial applications. It can be produced by *Aspergillus* spp. and *Penicillium* spp., especially *A. flavus*[1] and *A. parasiticus*[2]. Although kojic acid has been produced in industry, it is still extensively investigated. Two main approaches are the discovery and development of effective strains and the development of the fermentation process[1]. Presently, several potential kojic acid producing strains have been isolated. However, attention on the improvement of strains, for example via mutation or genetic engineering techniques, has been limited[1].

Various carbon sources (glucose, xylose, sucrose, starch, maltose, lactose or fructose) have been used as substrates for kojic acid production[3–6]. The highest kojic acid production of 39.90 g/l in submerged batch fermentation using *A. flavus* Link 44-1 was obtained when using glucose as a substrate[4]. Using corn starch, rather than glucose, was reported to yield a better production of kojic acid using *A. flavus* S33-2[7]. Agroindustry by-products have also been used as a substrate for kojic acid production, the culture of *A. flavus* number 7 grown on molasses medium successfully produced kojic acid with a maximum level of 53.5 g/l after 8 days of incubation[5]. The application of alternative substrates, especially low cost or easily available materials instead of glucose, is one of the important approaches to improve production yield and reduce the cost of kojic acid production.

Different techniques were applied to increase the production of kojic acid, such as repeated fed-batch membrane-surface liquid culture[8] and cell

immobilization[9]. Furthermore, the development in biotransformation processes is still required for effective production of kojic acid.

Kojic acid is a well-known antityrosinase agent, which has been efficiently used as whitening or lightening agents and UV protectants in cosmetics, also widely used to treat hyperpigmentation and wrinkles due to its ability to scavenge free radicals as antioxidant[10]. In pharmaceutics, kojic acid is used for the treatment of pain and inflammation[11]. It has also been used in food, agriculture and chemistry applications[1]. It acts as a preservative to prolong the shelf-life of products against both chemical and microbial degradation[3,12]. Health foods containing kojic acid are widely marketed in Japan[13].

Antioxidants are molecules that inhibit the oxidation of other molecules. Antioxidant activity is the total capacity of antioxidants for eliminating free radicals[14]. Antioxidants play a significant role as health protecting factor. Various investigations suggest that antioxidants reduce the risk for chronic diseases including cancer, heart disease[15]. Furthermore, the use of antioxidants is an effective approach to prevent symptoms related to photo-induced ageing of the skin[16].

Melanin is essential for protecting human skin against radiation, but the accumulation of abnormal melanin induces various pigmentation disorders, such as freckles and melasma[17]. Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase enzyme[18] that catalyses melanin synthesis, oxidises tyrosine to dopa, and dopa to dopaquinone[19]. Thus inhibition of tyrosinase activity is applicable for skin-lightening. Furthermore, melanin synthesis involves oxidation reactions and the generation of superoxide anions (O_2) and hydrogen peroxide (H_2O_2) through the l-tyrosine-tyrosinase reaction. It is well known that reactive oxygen species (ROS) play an important role in the regulation of melanocyte proliferation and melanogenesis[20]. Accumulation of oxidative damage to macromolecules (lipids, DNA, and proteins) by reactive oxygen and nitrogen species (RONS) resulted in ageing[21]. Thus ROS scavengers and inhibitors may down-regulate hyperpigmentation and UV-induced melanogenesis[20].

At present, there are various cosmetic products containing bacteria and yeast probiotics, such as aftershaves, anti-ageing serums, face and body lotions, toothpaste, sanitary napkins, shampoos and oral care gums[22]. However, new approaches in cosmetic development by adding fermented broth to cosmetic products, especially for reducing melanogenesis and anti-ageing, still present a challenge to be overcome.

In this study, we aimed to investigate the production of a fermented mixture containing kojic acid from the fermentation of rice as a carbon source, which was readily available. We compared the use of mixed or separated cultures of *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259 in the fermentation. Free radical scavenging and antityrosinase activity of the fermented mixture, were evaluated.

MATERIALS AND METHODS

Strains and chemicals

Amylomyces rouxii TISTR 3182 and *Aspergillus oryzae* TISTR 3259 were purchased from the Thailand Institute of Scientific and Technological Research (TISTR, Pathum Thani, Thailand). Standard kojic acid was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), glucose and yeast extract were purchased from HiMedia Laboratories (Mumbai, India) and other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of medium and stock cell suspensions

Both *A. rouxii* and *A. oryzae* were maintained on potato dextrose agar (PDA) slants and kept at 27 ± 2 °C for 3 days for *A. rouxii* and 7 days for *A. oryzae*, respectively. The medium for cell growth and biotransformation contained glucose 100, yeast extract 2.5, KH_2PO_4 1, KCl 0.5 and $MgSO_4 \cdot 7H_2O$ 0.5 g/l in deionized water. The pH was adjusted to 4.0 prior to sterilization at 121 °C for 20 min.

To prepare seed culture, cultures on PDA slants were scrabbled and fungus spores were rinsed with 0.01% (v/v) tween 80 in 0.9% (w/v) NaCl solution, followed by dilution for inoculation. One millilitre of the spore suspension (1×10^6 spore/ml) was inoculated into 250 ml-Erlenmeyer flasks containing 100 ml of biotransformation medium and cultured under conditions at 28 ± 2 °C, 220 rpm for 24 h on an orbital shaker (Revco Scientific Inc., Asheville, NC, USA).

Production of kojic acid from glucose

Sterile biotransformation medium with an initial volume of 90 ml in 250 ml-Erlenmeyer flasks was inoculated with 10 ml seed culture of *A. oryzae* and cultured under conditions at 27 ± 2 °C with occasional shaking for 15 days. During the biotransformation process, 1 ml of the culture medium was

collected at 0, 3, 4, 6, 7, 10, 12, and 15 days for analysis of kojic acid and glucose.

Production of kojic acid from rice

To investigate the production of kojic acid from rice, steamed rice was used as a carbon source instead of glucose. The initial volume of 80 ml sterile biotransformation medium with 100 g/l of rice in 250 ml-Erlenmeyer flasks was inoculated with 10 ml seed culture of *A. rouxii* and cultured at 27 ± 2 °C with occasional shaking. After cultivation for 3 days, a seed culture of *A. oryzae* of 10 ml was inoculated into the medium and cultured under the same conditions. The biotransformation medium was collected at 0, 3, 4, 6, 7, 10, 12, and 15 days for determination of kojic acid and glucose.

In addition, we investigated the production of kojic acid from rice by separation of *A. rouxii* mycelium from the culture medium before inoculation of *A. oryzae*, and then cultured under the same condition. The biotransformation medium was collected at the same interval time for analysis of kojic acid and glucose.

Determination of kojic acid by the colorimetric method

The collected fermented broth was centrifuged at 2000 rpm and filtered through a 0.45 μm membrane filter before analysis. Quantitative analysis of kojic acid in the culture was determined using a colorimetric reaction between kojic acid and $FeCl_3$, previously described by Bentley[23], with a slight, small-scale, modification. 50 μl of tested sample or medium (blank) was mixed with 250 μl of 1% (w/v) $FeCl_3$ in 0.1 N HCl in a 96-well plate. The resulting purple solution was measured by a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 504 nm and the kojic acid content was calculated. The calibration curve of kojic acid standard solution at concentrations between 10 and 80 mg/l showed good linearity. The regression line for kojic acid was described by the following: $Y = 0.00017X - 0.0148$ ($R^2 = 0.9997$), where Y is the absorbance and X is the concentration of kojic acid (mg/l).

Determination of glucose by the dinitrosalicylic acid (DNS) method

Glucose was determined by the dinitrosalicylic acid (DNS) method, with a slight, small-scale, modification[24]. Each 25 μl of tested sample or distilled water (blank) and DNS reagent was added into a heat resistant 96-well plate with a cap and mixed well. The microtitre plate was placed in the oven for 10 min to perform the reaction and then placed on ice to stop the reaction. 250 μl of 40% (w/v) of potassium sodium tartrate (Rochelle salt) solution was added to each well before measurement of glucose content by a microplate reader at 575 nm. The calibration curve of a glucose solution at concentrations between 200 and 1000 mg/l showed good linearity. The regression line for glucose was described by the following: $Y = 0.0004X - 0.0422$ ($R^2 = 0.9993$), where Y is the absorbance and X is the concentration of glucose (mg/l).

Antioxidant activity of fermented broth by the DPPH scavenging activity assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of the fermented broth which gave the maximum yield of kojic acid was determined by a modified method, as previously described[25]. The fermented broth was centrifuged, filtered through a 0.45 μm membrane filter and diluted with distilled water to prepare 10, 50, and 100% (v/v) solutions. Fifty microlitres of different concentrations of tested samples were mixed with 150 μl of 0.1 mM DPPH in ethanol in each well of a 96-well plate. The mixture was allowed to stand for 30 min at 27 ± 2 °C, protected from light. When DPPH was reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine, the absorbance at 515 nm was measured by the microplate reader (Eon, BioTek, Winooski, VY, USA). Lower absorbance of the reaction mixture indicated higher free radical activity[26]. Distilled water was used as a blank, while kojic acid (1.76, 3.52, 7.04 mM) and ascorbic acid (0.57 mM) were used as positive controls. The experiments were done in triplicate. The percentages of DPPH radical-scavenging activity were calculated as follows:

$$\%\,\text{Inhibition} = \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{blank}}}\right) \times 100$$

Antityrosinase activity of fermented broth by the dopachrome method

Tyrosinase inhibitory activity of the fermented broth which gave the maximum yield of kojic acid was measured using the dopachrome method[27], with a slight modification. Forty microlitres of each concentration of tested samples (10, 50, and 100% (v/v)) and 120 μl of 8.0 mM L-dopa in 67 mM of phosphate buffer (pH 6.8) was added to a 96-well plate, followed by 120 μl of 125 U/ml mushroom tyrosinase enzyme. The assay mixture was incubated for 20 min at 37 °C. After that the formation

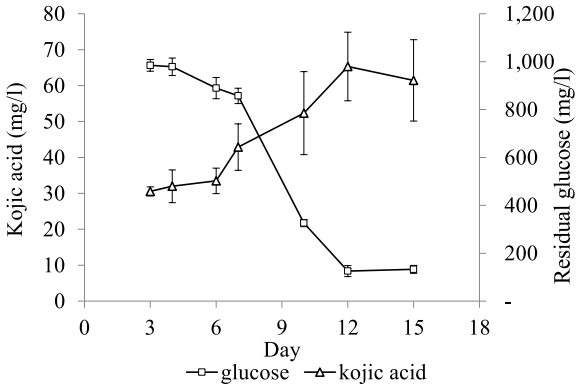

Fig. 1 Fermentation of glucose for kojic acid production by *A. oryzae* TISTR 3259. Results are represented as mean ± SE ($n = 3$).

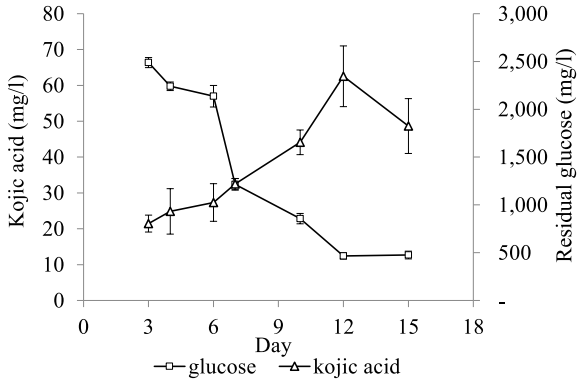

Fig. 2 Fermentation of rice for kojic acid production by mixed culture of *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259. Results are represented as mean ± SE ($n = 3$).

of dopachrome in the reaction mixture was determined by spectrophotometric analysis at 490 nm using a microplate reader. Distilled water was used as a blank, while kojic acid (1.76, 3.52, 7.04 mM) and ascorbic acid (0.57 mM) were used as positive controls. The experiments were done in triplicate. The percentages of tyrosinase inhibition were calculated as follows:

$$\%\,\text{Inhibition} = \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{blank}}}\right) \times 100.$$

RESULTS AND DISCUSSION

Production of kojic acid from glucose

Glucose was used as carbon source for kojic acid production by *A. oryzae* TISTR 3259. As shown in Fig. 1, the results showed that *A. oryzae* TISTR 3259 can produce kojic acid from glucose. More than 90% of glucose was consumed by the culture on day 12, while kojic acid increased rapidly after 6 days of fermentation. The maximum yield of kojic acid was 65.3 ± 9.6 mg/l at day 12 of fermentation. This was in accordance with the previous study which reported that glucose was the best carbon source for kojic acid production by *A. oryzae*[3]. During the biotransformation process, kojic acid is generated from glucose through a multistep cell-bound enzyme system[3].

Production of kojic acid from rice

As shown in Fig. 2, rice (*Oryza sativa*) was used as a carbon source instead of glucose for the production of kojic acid by *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259. In rice medium with mixed cultures of both strains, more than 90% of glucose was consumed by the culture at day 12, while kojic acid production exhibited a rapid increment after 6 days of fermentation. Kojic acid was gradually detected, reaching a maximum yield of 62.5 ± 8.5 mg/l at day 12 of fermentation. The result showed that rice could be used as a carbon source for kojic acid production by mixed cultures of *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259. In the control group (no glucose or rice added), we also detected a small amount of kojic acid in the culture medium (data not shown). This may be due to some glucose in seed culture that *A. oryzae* TISTR 3259 can use as a carbon source to produce kojic acid.

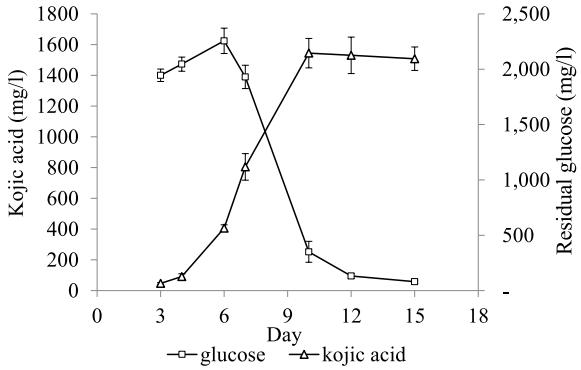

Fig. 3 Fermentation of rice for kojic acid production by separated culture of *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259. Results are represented as mean ± SE ($n = 3$).

Rice is primarily composed of carbohydrates, made up of long chains of glucose known as amylose and amylopectin[28]. Rice can be converted into glucose by enzymatic hydrolysis. *A. rouxii* and *A. oryzae* are filamentous fungi which have

been widely used commercially in the production of fermented foods[29,30]. They have been reported to produce glucoamylase which catalyses the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules[31], thereby resulting in glucose production[30]. Furthermore, the production of kojic acid from rice by separation of *A. rouxii* TISTR 3182 mycelium from the culture medium before inoculation of *A. oryzae* TISTR 3259 was investigated. As shown in Fig. 3, glucose consumption was similar to that in rice medium with mixed cultures, but kojic acid concentration obtained from this culture increased to a maximum yield of 1.54 ± 0.10 g/l at day 10, which was significantly higher than that obtained from mixed cultures. Although the previous study reported that metabolic synergisms among fungi can be achieved using mixed cultures in the solid-state fermentation process[32]. However, we have found that the growth of both *A. rouxii* TISTR 3182 and *A. oryzae* TISTR 3259 in the mixed culture might interfere with each other and resulted in a lower production of kojic acid in this study.

Our results revealed that kojic acid yield in the medium containing glucose or rice reached the maximum yield within 10–12 days of fermentation, and then declined with increasing time. This gradual reduction of kojic acid concentration in glucose and rice medium was in accordance with previous reports[33,34]. It has been postulated that this may be due to the degradation of kojic acid to oxalic and acetic acid by the mycelium under glucose depleted conditions[28,35]. Furthermore, there might be a slight deactivation of the enzymes involved in kojic acid biosynthesis during prolonged incubation[28].

Different production yield of kojic acid by using optimized production process, medium, substrates and efficient microbial strains have been reported. The application of alternative substrates, especially low cost or easily available materials instead of glucose, such as rice, agro products and agroindustry wastes or by-products is one of the important approaches to improve production yield and reduce the cost of kojic acid production.

Antioxidant activity of fermented broth

The DPPH assay has been widely used to evaluate the free radical-scavenging activity of various antioxidants. DPPH is a stable nitrogen-based free radical with a violet colour. After reduction by either hydrogen- or electron-transfer, the colour converts to yellow. Substances which are able to execute this reaction can be considered as antioxidants[36]. As

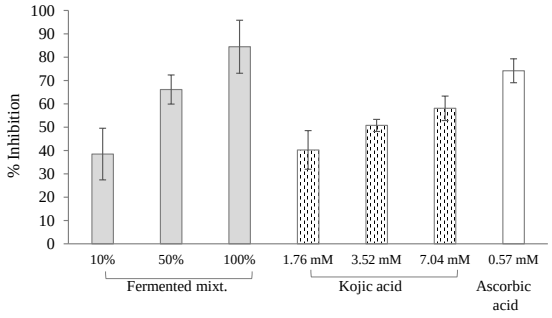

Fig. 4 DPPH radical-scavenging activity of fermented mixtures, as compared to kojic acid and ascorbic acid. Results are represented as mean ± SE ($n = 3$).

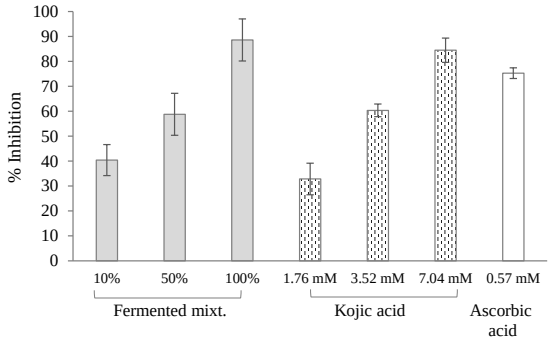

Fig. 5 Tyrosinase inhibitory activity of fermented mixtures, as compared to kojic acid and ascorbic acid. Results are represented as mean ± SE ($n = 3$).

shown in Fig. 4, DPPH radical-scavenging activity of fermented mixture from rice medium (in separated cultures) increased in a dose-dependent manner. The maximum radical-scavenging activity of the fermented mixtures was $84 \pm 11\%$ at the maximum tested concentration, which was higher than both ascorbic acid and kojic acid at the concentrations tested. However, the potency of the fermented mixture was similar to kojic acid when comparable concentrations were used. The previous investigation revealed that sap sample from white plain rice and various pigmented rice contained gamma-linolenic acid and linoleic acid[37]. These unsaturated fatty acids might be responsible for free radical-scavenging activity. Pigmented rice such as purple rice, red rice and black rice might be applied for our further investigation to increase antioxidant activity of the fermented product[35].

Antityrosinase activity of fermented broth

As shown in Fig. 5, the mushroom tyrosinase inhibitory activities of the fermented mixture increased in a dose-dependent fashion. The maximum inhibition was 89 ± 11% at the maximum tested concentration, which was higher than both ascorbic acid and kojic acid at the concentrations tested. However, the potency of the fermented mixture was slightly lower than kojic acid when comparable concentrations were used. The previous study reported that *A. oryzae*-fermented rice bran extracts contained various organic acids, including oxalic acid, citric acid, succinic acid, kojic acid and acetic acid[32]. However, some organic acids, such as glycolic acid and lactic acid, could directly inhibit tyrosinase activity during melanin synthesis in melanoma cells[38]. Accordingly, lactic acid in *Lactobacillus rhamnosus* spent culture supernatant suppressed tyrosinase activity in vitro in a dose-dependent manner[39]. Hence it is possible that the presence of some organic acids and other compounds in the fermented mixture decreased tyrosinase activity in our study.

CONCLUSION

The present study revealed that rice was a practical and attractive carbon source for the production of fermented mixtures containing kojic acid. *Amylomyces rouxii* TISTR 3182 and *Aspergillus oryzae* TISTR 3259 could be promising strains for kojic acid production using rice as substrate. Rice production has played a critical role in Thailand's socio-economic development. Hence it is easily available and could support a continuous production line as a raw material in the future. Different rice varieties or cereals might be further investigated to produce fermented mixtures containing kojic acid as value-added agriculture products. Furthermore, our results suggested that the fermented mixtures may have the potential to be further developed as a functional food and cosmetic ingredient, with antioxidant and whitening effects.

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