Faculty of Resource Science and Technology

Screening of Kojic Acid Producing Fungi from UNIMAS Fungal Collection and Production of Kojic Acid

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Screening of Kojic Acid Producing Fungi from UNIMAS Fungal Collection and Production of Kojic Acid

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A final project report submitted in partial fulfillment of the Final Year Project II (STF 3015) course

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May ALLAH bless and grant you happiness ever after. Amin.

Declaration

I, Siti Aisha Na'illa bt Che Musa declare that this thesis is my own work and effort that it has not been submitted anywhere any award. Where other sources of information have been used, they have been acknowledged.

Signature:…….

Date:……

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List of Abbreviations

cm	Centimeter
DNS	Dinitrosalicyclic acid
FDA	Food and Drug Administration
g	Gram
g/L	Gram per Liter
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
M	Molarity
MEA	Malt Extract Agar
mL	Milliliter
N	Normality
NaOH	Sodium Hydroxide
nm	Nanometer
sp	Species
UV	Ultraviolet
(v/v)	Volume per Volume
(w/v)	Weight per Volume
P_{max}	Maximum production
X_{max}	Maximum cell

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Screening of kojic acid producing fungi from UNIMAS Fungal Collection and production of kojic acid

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ABSTRACT

Kojic acid is a secondary metabolite produced mainly by *Aspergillus* species. Due to high demand and wide applications of this product, many research has been done in order to produce higher yield of this product. In this present work, via submerged fermentation, the result of screening process showed only one strain out of eight strains being tested produced kojic acid which is *Aspergillus flavus* NSH9. Kojic acid production by *A. favus* NSH9 was optimized under glucose concentration which is 110 g/L, pH 4 and 10^7 spore concentration and the highest kojic acid yield obtained are 15.38 g/L, 30.44 g/L, and 34.06 g/L respectively. Other than that, the incubation time for the kojic acid production at inoculums size namely 10^5 and 10^7 spores/mL were at day 22 and 26 respectively were also determined in this study. This result indicates the potential of *A. flavus* NSH9 as one of the producing fungi in this field.

Keywords: *Aspergillus flavus* NSH9, Kojic acid, Optimization, Screening, UNIMAS Fungal Collection

ABSTRAK

Kojik asid merupakan produk metabolisma kedua yang dihasilkan oleh spesis Aspergillus. Oleh sebab produk ini mendapat permintaan yang tinggi dan penggunaan yang meluas, banyak kajian yang telah dijalankan untuk meningkatkan pengeluaran produk ini. Dalam kajian ini, melalui fermentasi cecair keputusan kajian mendapati hanya satu strain yang berjaya menghasilkan kojik asid iaitu Aspergillus flavus NSH9. Penghasilan kojik asid dari A. flavus NSH9 kemudian di optimumkan dengan mengaplikasikan kepekatan glukosa iaitu pada 110 g/L, pH 4 dan kepekatan spora 10^7 spora/mL dimana kepekatan kojik asid tertinggi yang diperolehi adalah seperti berikut; 15.38 g/L, 30.44 g/L, dan 34.06 g/L. Masa inkubasi untuk penghasilan kojik asid juga dapat ditentukan dimana pada kepekatan spora yang berlainan iaitu 10^5 dan 10^7 spora/mL ialah pada hari ke 22 dan 26. Keputusan dari kajian menunjukkan A. flavus NSH9 berpotensi untuk menjadi salah satu kulat dalam penghasilan kojik asid.

Kata kunci: *Aspergillus flavus* NSH9, Kojik asid, Optimum, Pemerikasaan, Koleksi Kulat UNIMAS

1.0 Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is an organic acid. It was originally isolated in Japan by Saito in 1907 from mycelia of *Aspergillus oryzae* grown on steamed rice (El-Aasar, 2006). The name of "kojic acid" was derived from "Koji" the fungus starter use in oriental food fermentations. Kojic acid is produced by different types of fungal like *Aspergillus* species *Penicillium* species (Rosfarizan*et al.,* 2010) and *Acetobacter* species (Pickut, 2011) as their secondary metabolites products. It is produced biologically by aerobic fermentation process (Rosfarizan *et al.,* 2010).

It has wide variety of applications especially in cosmetic and healthcare industries like as material for the production of skin whitening creams, skin protective lotions, whitening soaps and tooth care products (Rosfarizan *et al.*, 2010). Other than that, kojic acid also has many applications in the field of food, medical, agricultural, chemical and many more.

The high demand of the kojic acid this recent decades shows that the production of this organic acid must be increase. This can be seen when the U.S. Food and Drug Administration (FDA) has banned on over-the-counter sales of cosmetic products containing hydroquinone, on August 29, 2006 (Rosfarizan*et al.,* 2010). Until 2000, production of kojic acid was manufactured by two companies in China, and each company in Japan, Switzerland and USA (CIS Information Services, 2000).

The research on a rodent shows that the consumption of hydroquinone products can cause skin cancer makes the industry people change to use kojic acid to replace the hydroquinone. Based on the information available from IARC Monographs Volume 79, there is inadequate evidence in human for carcinogenicity and limited evidence in experimental

animals for carcinogenicity. This give the overall evalution that kojic acid is not classified as danger.

Thus, the study on various fungi is seen could be helped in order to fulfill the demands and supply the products. The production of this extracellular secondary metabolite is influenced by many factors such as nutrient medium, culture condition, cell concentration and the growth rate (Rosfarizan, 2000). These factors must be well investigated in order to produce high yield of quality kojic acid in less time.

"Screening of kojic acid producing fungi from UNIMAS Fungal collection and production of kojic acid" is a preliminary study that was conducted in order to study the availability of UNIMAS fungal o produce kojic acid. Eight fungi strains obtained from Molecular Genetic Laboratory, Department of Molecular Biology, Universiti Malaysia Sarawak (UNIMAS) were used in this study. This study was investigated the capability of eight indigenous different fungi strains to produce kojic acid. The best selected kojic acid producer then undergo investigation in order to give higher kojic acid production under optimum conditions to be applied.

In this study, the fungi used are the fungi from UNIMAS Fungal Collection. There are eight strains of fungi that will be used which are *Aspergillus flavus* NSH9, *Aspergillus niger*, *Aspergillus versicolor*, *Penicillium chermisenum*, *Trichoderma virens*, *Trichoderma harzianum*, *Marasmius cladophyllus* and *Bionecteria ochroleuca.*

There were two main objectives of this study are as follows:

1. To screen for the kojic acid producing ability among different strains of indigenous fungi from UNIMAS Fungal Collection.
2. To optimize several parameters that produce high kojic acid using the best selected kojic acid producing fungi sought in the first stage. The parameters include, effects of kojic acid production in different glucose concentration, different initial pH and different inoculums size.

2.0 Literature review

Kojic acid has been identified as an antioxidant especially in cosmetic products and it is also widely used in the food product. Burnett *et al*., (2010) reported that this compound was not a toxicant in acute, chronic, reproductive, and genotoxicity studies. Other than that, the safety level of the kojic acid concentration to be used in the cosmetic products also must be not more than 1% as underlined by the Cosmetic ingredient review (CIR) Expert Panel (Burnett *et al.*, 2010). Figure 1 shows the kojic acid chemical structure where thw structure is more or less to glucose structure.

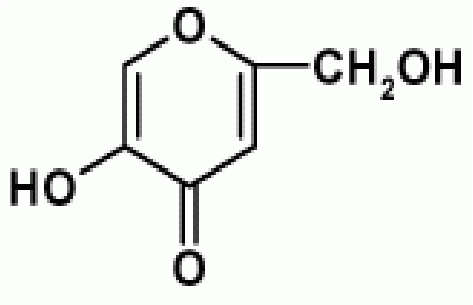

Figure 1 Kojic acid chemical structure

The application of kojic acid as antifungal in certain commercial products either in agricultural or veterinary situations also shows its promising activities in enhancing drugs and fungicides for fungi control. According to Kim *et al*., (2012) kojic acid acts as antifungal agent by targeting the fungal antioxidative system and also act as a chemo sensitizing enhancer of antimycotic activity.

Table 1 below shows the amount of kojic acid used in various applications.

Table 1 Amount of kojic acid contents in production of food and cosmetics.

Amount (%)	Applications (IARC Monographs Volume 79)
0.2	Flavourings
1	Anti-depigmenting for agricultural products)
0.1	Flour production
0.2	Meat production
0.05	Syrup production
0.5	Whitening agent in cosmetics production

2.1 Kojic acid producing microorganisms

2.1.1 *Aspergillus* species

Kojic acid is produced mainly by *Aspergillus* species. Since 1907, Saito discovered the production of kojic acid by *A. oryzae* grown on steamed rice. Based on Rosfarizan *et al.,* (2000) other than *A. oryzae* (El-Aasar, 2006), *A. flavus* and *A. tamari* also could produced large amount of kojic acid. Among the *Aspergillus* species fermentation of *A. flavus* was reported to produce high yield of kojic acid (Bajpai, 1982) followed by *A. oryzae* (Rosfarizan, 2000) as reported by Kitada *et al.* (1967).

El- Aasar, (2006) also reported that *A. parasiticus* is another *Aspergillus* species that can contribute to the production of kojic acid under after optimization of growth fermentation and productivity. Parrish *et al.,* (1965) reported that out of 14 species of *Aspergillus* nine species of *Aspergillus* can produce kojic acid. The nine species of *Aspergillus* reported to have the ability to produce kojic acid are; *A. clavatus*, *A. effuses*, *A.flavus*, *A. nidulans*, *A. oryzae*, *A. parasiticus*, *A. tamarii* and *A. ustus*.

Based on Alverson (2003), Kobe *et al.,* (1977) and Wilson (1966) reported that *A. niger* can produce variety of mycotoxin including oxalic acid and kojic acid. However, Schuster *et al.,* (2002) reported that ochratoxin is the only mycotoxin produced from *A. niger* as cited in Nielsen, (2003). There are more research have been done on oxalic acid (Gupta and Mukerji, 2001) but less on the ability of producing kojic acid by *A. niger*.

2.1.2 *Penicillium* species

Penicillium species reported to have the ability to produce kojic acid (Rosfarizan *et al.*, 2010). Parrish *et al.,* (1965) also reported out of seven species of *Penicillium* four species of *Penicillium* can produce kojic acid. The four of the *Penicillium* species includes *P.citrinum*, *P. griseofulvum*, *P. purpurogenum* and *P. rubrum*. However, no investigation has been found on kojic acid production by *P. chermesinum*.

2.1.3 *Trichoderma* species

Saleh *et al.,* (2011) reported that most of the *Trichoderma* species have antibacterial activity. Based on the research, there were six *Trichoderma* species that have been investigated which are; *T. V6, T. pseudokoningii, T. reesei, T. viridae, T. harzianum* and *T. virens*. Two best species were *T. viridae* and *T. reesei* which gave the highest antibacterial activity against almost all of the human pathogenic bacteria. It was discovered the antibacterial compound contain kojic acid as their main compound.

According to the research, the screening of kojic acid and reducing sugar during the growth of *T. viridae* and *T. reesei* increase with the decreasing reducing sugar during the incubation time. The result shows that the fungi have the enzymatic system responsible for the production of kojic acid using glucose as carbon source (Saleh *et al*., 2011). However, *T. harzianum* and *T. virens* are less reported in the research. Since both of the species also found to have antibacterial compound that able to against human pathogen it provides a chance to become one of the kojic acid producing fungi.

2.1.4 Other species

However, the production of kojic acid by other species of fungi that will be used in this study, namely *A. versicolor, P. chermesinum, M. cladophyllus* and *B. ochroleuca* have not been reported in any literature review yet.

2.2 Kojic acid production

Kojic acid is produced mainly by *Aspergillus* species, but the commercial production is performed by *A. oryzae* and *A. flavus* using glucose as carbon substrate (Christian *et al.,* 2002). *A. tamari, A. oryzae, A. albus* and *P. puberulum* are also examples of kojic acid producing fungi (Sahasrabudhe and Sankpal, 2001).

2.2.1 Biosynthesis of kojic acid

Based on earlier study by Arnstein and Bentley (1953), the production of kojic acid is by direct conversion of glucose to kojic acid by two processes without cleaving the structure of glucose. The processes involved were oxidation and dehydration. The glucose was first oxidized to 3-ketoglucose and subsequently dehydrate to kojic acid. However, this pathway was only applied to glucose that have 6 carbons but not to the compound that have less than 6 carbons.

Later, Bajpai *et al.,* (1981) reported that there are enzymes involved in the production of kojic acid. The enzymes found presence in the mycelia of *A. flavus* are hexokinase, glucose-6-phosphate dehydrogenase, glucose dehydrogenase and gluconate dehydrogenate. However, among the enzymes stated above, the most enzymes detected involved in conversion of glucose to kojic acid are glucose dehydrogenase and gluconate dehydrogenate (Bajpai *et al.,* 1981).

2.2.2 Carbon source

Carbon source plays the most important role in kojic acid production since it supplies the energy for the cell and as substrate for kojic acid production. Carbon sources also functions as biosynthesis of cellular constituents like carbohydrates proteins, lipids, nucleic acids and many more. Kojic acid producing do not require specific carbon sources since all of the cultures are able to utilize various carbon sources like glucose, fructose, sucrose, maltose, and mixture of glucose and sucrose (Sahasrabudhe and Sankpal, 2001). Based on what have been reported by Kitada *et al.*, (1967) the highest yield of kojic acid produced by *A. oryzae* was obtained in the fermentation using glucose as carbon source, followed by sucrose and fructose. The fermentation using other carbon source like starch and xylose gave high growth of the fungi but no kojic acid detected.

Rosfarizan and Ariff, (2000) investigated on the effect of using different types and concentration of carbon sources on kojic acid production by *A. flavus* where the types of carbon sources that have been tested included glucose, xylose, sucrose, starch, maltose, lactose and fructose. Result obtained showed glucose as the best carbon source. Glucose appeared to give the highest yield of kojic acid (0.989 g kojic acid g carbon^{-1}). El-Aasar, *(*2006) reported that glucose also have yield highest kojic acid production by *A. parasiticus* and followed by sucrose and beet molasses. Thus, glucose is suggested to be used in order to screen the capable kojic acid producing fungi since it is the simplest sugar and can be utilize in most of the organism as their carbon sources (Black, 2007).

Concentration of carbon source also affects the production of kojic acid. Rosfarizan and Ariff, (2000) reported that the highest kojic acid production by *A. flavus* was obtained with 100 g/L glucose. Their research also came out with the inhibition of kojic acid

production when the glucose concentration is high (150- 200 g/L). Besides, it is also reported that the concentration of carbon source or sugar resulted in significant increase in residual sugar. The residues is due to the inability of fungi to metabolize high levels if sugar (El-Aasar, 2006). However, lower glucose concentration (50-80 g/L) will produce lower kojic acid production where the glucose was consumed for the cell growth. Thus, the production of kojic acid can be range in between 80 g/L to 150 g/L in order to look for optimization. Table 2 shows the types of carbon sources used and the optimum concentration of the carbon source with the concentration of kojic acid produced.

Table 2 Types of carbon sources used and their optimum concentration with the concentration of kojic acid produced.

Carbon sources	Concentration of carbon sources (w/v)	Microorganism	Kojic acid Concentration (g/L)	References
Glucose	6%	*A.parasiticus*	43	El-Aasar (2006)
Sucrose	4%	*A.parasiticus*	20	El-Aasar (2006)
Beet molasses	6%	*A.flavus*	20	El-Aasar (2006)
Glucose	10%	*A.flavus*	39.9	Rosfarizan & Ariff (2000)
Xylose	10%	*A.flavus*	35.1	Rosfarizan & Ariff (2000)
Sucrose	10%	*A.flavus*	22.98	Rosfarizan & Ariff (2000)

2.2.3 pH

pH condition of the fermentation reaction also give big effect to the growth and production of kojic acid by fungi. The research of pH usually based on initial pH of the culture during fermentation process. Several fungi like *A. oryzae* and *A. flavus* have the ability to produce kojic acid at pH range of 3 to 7 (Rosfarizan *et al.*, 2000). For kojic acid production by *A. flavus*, Rosfarizan *et al.*, (2000) reported that the optimal pH is at pH 3.

Based on the research, pH 6 to pH 7 yield highest growth of the cell but kojic acid only produced at pH 3. However, at pH 2 the growth of the culture and production of kojic acid are greatly inhibited. The effects of initial pH on kojic acid production by *A. parasiticus* reported that the optimum pH is 4.5 and 6.2 by Lin *et al.*, (1976). El-Aasar (2006) reported that optimal condition of *A. parasiticus* is pH 5. However, when pH increased at 5.5, the production of kojic acid decreased. All of the literature review regarding initial pH control shows the range of pH that can induce the growth and kojic acid production is between pH 2 to 6. The optimal pH is very important to be determined since it can greatly affected the optimum production of enzymes required for kojic acid production. Table 4 shows the optimum pH used and the concentration of kojic acid produced by *Aspergillus* species.

Table 3 Optimum pH for kojic acid fermentation by *Aspergillus* species.

Optimum pH	Species	Kojic acid concentration (g/L)	References
pH 5	*A. parasiticus*	34.38g/L	El-Aasar, 2006
pH 3	*A. flavus*	30.20 g/L	Rosfarizan & Ariff, 2000

3.0 Materials and Method

The research work was divided into two stages, namely screening of potential kojic acid producer and optimization of kojic acid fermentation. In the first stage, the best kojic acid producing fungal strain was identified. Meanwhile, in the second stage, optimization of kojic acid fermentation by the potential kojic acid producer sought in earlier stage was carried out. The effects of three parameters; glucose concentration, inoculum concentration and initial pH on kojic acid fermentation were investigated.

3.1 Screening of potential kojic acid producer

3.1.1 Microorganisms preparation

Eight strains of fungi from UNIMAS Fungal Collection were used in this study. The fungi were *A. flavus*, *A. niger*, *A. versicolor*, *P. chermesinum*, *T. virens*, *T. harzianum*, *M. cladophyllus* and *B. ochroleuca* which were obtained from Department of Molecular Biology, Universiti Malaysia Sarawak (UNIMAS). The fungi were grown on Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) plates for ten days at 29°C and were subcultured for working culture purpose. The working cultures were maintained.

In the first stage, the inoculums used were in the form of blocks or plug with standardized size. Blocks or plugs were used by cutting the ten-day old cultures grown on PDA and MEA in approximately 1 cm X 1 cm size. Meanwhile in the second stage, the inoculum used was in the form of spore suspension. Spore was harvested using sterile 0.001% (v/v) Tween-80. The standardization of inoculum was based on spectrometry method

(Petrikkou *et al.*, 2001) whereby the absorbance of the spore suspension was translated into equivalent spore concentration based on the standard curve developed for the strain.

3.1.2 Media preparation

The same optimized medium by Madihah *et al.*, (1996) for kojic acid production was used. The medium consists of % (w/v); glucose, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05; yeast extract 0.5 and methanol 0.4 (v/v). The working volume for every culture was 150 mL.

3.1.3 Standard curve preparation

There are three types of standard curve that have been prepared which are; standard curve of spore concentration, standard curve of residual sugar analysis which is glucose concentration, and standard curve of kojic acid concentration. The graph then plotted for determination of spore concentration , glucose concentration, and kojic acid concentration.

3.1.3.1 Spore concentration standard curve

Spore concentration standard curve was developed by count the harvested spore using Tween-80 on haemocytometer. The different concentrations of spore suspensions were measured by absorbance reading at 550 nm wavelength. The graph of absorbance of the spore suspension versus the concentration of spore was plotted. The equation was obtained from the standard curve for determination of spore concentration.

3.1.3.2 Residual sugar analysis standard curve

Glucose concentration was determined by DNS method by Miller (1957). The different concentrations of glucose were measured by absorbance reading at 575 nm wavelength. The graph of absorbance reading against different concentration of glucose was plotted. The equation was obtained from the standard curve for determination of glucose concentration.

3.1.3.3 Kojic acid standard curve

Kojic acid standard curve was developed by using different concentration of commercial kojic acid by Sigma Chemical Company (United States). The different concentrations of kojic acid was determined by colorimetry method by Bentley (1957), and measured by absorbance reading at 500 nm wavelength. The graph of absorbance reading against different concentration of kojic acid was plotted. The equation was obtained from the standard curve for determination of kojic acid concentration.

3.1.4 Kojic acid fermentation

The feasibility of all the strains in producing kojic acid was screened by cultivating them in production medium as proposed by Madihah *et al.,* (1996). In the screening of kojic acid production seven days old cultures were used, where 3 plugs of 1cm X 1cm size of each strain were inoculated into sterile 150 mL of fermentation medium in 250 mL shake flask. pH of each medium was standardized to pH 4.0 by adding either 1.0 M HCl or 1.0 M NaOH. The flasks then were agitated at 30°C on a rotary shaker at 150 rev/min. All experiments were carried out in duplicate.