Purification and Characterization of Kojic Acid Produced From Pineapple Waste by *Aspergillus flavus* Link 44-1 via Solid Substrate Fermentation (SSF)

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DECLARATION

I declared that this project entitled "Purification and Characterization of Kojic Acid Produced From Pineapple Waste by *Aspergillus flavus* Link 44-1 via Solid Substrate Fermentation (SSF)" is the result of my own research except as cited in the references. This project has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature: ....

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LIST OF ABBREVIATIONS

PDA	Potato Dextrose Agar
SSF	Solid Substrate Fermentation
mL	milliliter
M	Molarity
^0C	Degree of celcius
Mm	Micrometer
g	Gram
H_2O	water
nm	Nanometer
g/L	Gram per Liter
v/w	Volume per weight

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ABSTRACT

Kojic acid is an organic acid that is highly demanded in cosmetic, medical, and food industries. The present study was aimed to purify and characterize of kojic acid produced from pineapple waste. Crystallization technique was applied in purifying the kojic acid. Subsequently, characterization was based on NMR, UV and HPLC analyses. The results showed that about 1.5% (w/w) kojic acid could be traced from NMR analysis while the HPLC results indicated the presence of approximately 0.025g/L kojic acid produced from 5 gram of pineapple waste. From this study, it can be suggested that crystallization technique should be enhanced with supplementary procedures in enhancing the quality of kojic acid produced from pineapple waste in future.

Keywords: *Aspergillus flavus*, characterization, kojic acid, pineapple waste, purification

ABSTRAK

Asid kojic ialah satu asid organik yang mempunyai permintaan yang tinggi dalam kosmetik, perubatan, dan industri makanan. Kajian menunjukkan bertujuan untuk menambahkan pengeluaran asid kojik dari sisa nanas. Teknik pengkristalan telah diaplikasikan dalam menjernihkan asid kojik. Kemudiannya, pencirian berdasarkan NMR, UV dan analisis HPLC dilakukan. Keputusan menunjukkan bahawa sejumlah 1.5% (w/w) asid kojik dikesan dengan menggunakan analisis NMR manakala keputusan HPLC menunjukkan kehadiran kira-kira 0.025g / L asid kojic yang dihasilkan daripada 5 gram sisa nanas. Daripada kajian ini, dicadangkan supaya teknik pengkristalan seharusnya ditingkatkan dengan beberapa prosedur tambahan dalam meningkatkan kualiti asid kojik yang dihasilkan daripada sisa nanas pada masa depan.

Keywords: *Aspergillus flavus*, pencirian, asid kojik, hampas nanas, penjernihan

1.0 INTRODUCTION

In Malaysia, pineapple is one of the important agriculture crops. It contributes to the economic growth although the industry is comparatively small compared to other major industries such as rubber and palm oil. Every year, pineapple canning industries in the country generate bulk amount of waste. The disposal of pineapple waste can cause tremendous environmental hazard due to high content of organic material and suspended solid (Buckle, 1989).

Employing agricultural wastes for bioconversion processes is becoming popular in these years due to their significant advantages both economically and environmentally. Various agro-industrial waste and by-product like orange peel (Martin *et al*., 2000), sugar cane bagasse (Silva *et al*., 2002) and other food processing wastes (Muthulakhsmi *et al*., 2011) which have been used as alternative substrates for production of enzymes and other metabolites either by solid substrate fermentation (SSF) or submerged fermentation (SmF). The type of bio-products produced is greatly depending on the microorganism used such as fungi and bacteria (Rosfarizan, 2000).

Kojic acid is one of the bioproducts that can be possibly initiated from pineapple waste using via SSF. It is mostly used in various industrial fields especially in cosmetic, medicine and agriculture. According to Rosfarizan (2000), kojic acid can be highly produced by *Aspergillus* species and *Penicillum* species. However, *Aspergillus flavus* has been recognized as the major producer of kojic acid.

Despite the ongoing development of kojic acid production, information on the use of agriculture residues such as pineapple waste as substrate for kojic acid production is still scarce. Rusley (2012) has reported that the viability of kojic acid production from pineapple waste and several parameters have been optimized for its production. However, more information is still needed in enhancing its production. Furthermore, little is still known on the techniques of purification and characterization of kojic acid. Therefore, the present works was tailored to intensify the development of kojic acid production by *A. flavus* Link 44-1 using pineapple waste as substrate via SSF. Therefore, the specific objectives were:

I. To produce kojic acid from pineapple waste by *A. flavus* Link 44-1 under SSF.

II. To purify the kojic acid produced from pineapple waste by *A. flavus* Link 44-1 via SSF.

III. To characterize the kojic acid produced from pineapple waste by *A. flavus* Link 44-1 via SSF.

2.0 LITERATURE REVIEW

2.1 Pineapple Waste

Pineapple industry is one of the oldest-based export-oriented industries in Malaysia. Malaysia was once ranked as one of the top 3 pineapple producers in the world in 60's and early 70's. Until now, it is remained as one of the main commodities in Malaysia either for domestic or export market.

Pineapple is normally consumed fresh or can be processed as canned pineapple or juice (Arshad *et al*., 2008). Despite its economic advantages, pineapple processing industry may impose serious negative impacts on the environment and rural communities. Apart from irresponsible agricultural practices that result in soil deterioration and erosion, disposal of waste from the industry may expose the environment to severe pollution due to high amount of organic materials and suspended solids contained in waste (Ahmed *et al*., 2004).

The residues from the pineapple processing industry is one of the potential wastes that can be used as a substrate for fermentation due to its high content of total sugar (approximately 83 g/L) and protein (6.40 g/L) (Nigam, 1999). From commercial perspective, recycling the wastes would reduce the cost of waste disposal for canneries. This is because in normal practice the wastes need to be treated before disposal in order to reduce the organic load to the environment (Imandi *et al*., 2008). Hence, adopting pineapple waste as alternative substrate for bioconversion process serves as another environmental friendly way to overcome the bulk accumulation of residues generated by the pineapple processing industry.

2.2 Kojic Acid

One of the bioproducts that can be possibly initiated from sugary residues like pineapple waste is kojic acid. Kojic acid is an organic acid biologically produced by different types of fungi during aerobic fermentation of various natural foods (Kitada *et al*., 1967; Ariff *et al*., 1996; Wakisaka *et al*., 1998; El-Aasar, 2006). It is globally applied as whitening agent, as UV light protector (Ohyama and Mashiyama, 1990; Noh *et al*., 2009) and as pigment formation blocker (Masse *et al*, 2001) in cosmetic industries for decades. It also possesses antibiotic properties against gram-negative and gram-positive microorganism (Kotani *et al*., 1976). Kojic acid shows antibacterial and antifungal effects on several species of microorganism in form of azidometalkojates. Moreover, it also shows certain insecticidal activity and employed as a chelating agent for insecticides production (Buchta, 1982; Dowd, 1988). In Japan, kojic acid is naturally distributed in traditional foods to give a special taste, colours and also flavours (Wood, 1998)

2.3 Kojic acid producer

Fungi are widely known as kojic acid producing organism of which *Penicillium* sp. and *Aspergillus* sp. are reported as the main producers. Species in section Flavi (*A. flavus*) occur in nature as saprophytes in the soil and on decaying plant material or as parasites on plants, insects, and animals (Scheidegger and Payne, 2003). Non-aflatoxigenic species such as *A. oryzae* and *A. sojae* have been widely used in industry for food fermentation or for the production of enzymes (Geiser *et al*., 1998). Among the *Aspergillus* sp., *flavus-oryzae-tamarii* group is regarded as the best kojic acid producer (Arnstein and Bently, 1953). According to El-Aziz (2013), the ability of 58 different strains of *Aspergillus*, *Mucor*, and *Penicillium* to form kojic acid has been studied and *A. flavus* was found to be the highly active one for kojic acid production. Several *Aspergillus sp*. have been reported for kojic acid production such as *A. parasiticus* (Nandan and Polasa, 1985; Coupland and Niehaus, 1987; El-Aasar, 2006), *A. oryzae* (Kwak and Rhee, 1992; Takamizawa *et al*., 1996), *A. tamarii* (Gould, 1938) and *A. flavus* (Basappa *et al*., 1970; Ariff *et al*., 1996). In this work, *A. flavus* Link 44-1 which was isolated from morning glory flower (Rosfarizan, 1998) was used for kojic acid fermentation.

2.4 Development of kojic acid fermentation

Kojic acid fermentation has been long established ever since in ancient times. Kojic acid was discovered in several oriental foods such as miso, soy sauce and sake (Wood, 1998; Rosfarizan *et al*., 2010). Ironically, even though it was found as a product of SSF, nonetheless in recent years, its production is more concentrated by SmF due to the convenient applicability of the method particularly at industrial level. Not much is yet known on the potentiality of various usable agricultural residues as substrates for kojic acid production via SSF.

2.5 Kojic acid production via SmF

The development of kojic acid fermentation via SmF span over years. To date, various studies on kojic acid fermentation via SmF have been debated in different perspectives. **Table 1** summarizes several works pertaining to kojic acid production via SmF in various scopes of study.

Table 1: Summary of several studies on kojic acid fermentation via SmF

Description	**References**
Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* Link	Bassapa *et al*. (1969)
Enhance kojic acid production by *Aspergillus flavus* Link 44-1 in growth medium containing methanol	Madihah *et al*. (1996)
Isolation of a kojic acid-producing fungus capable of using starch as a carbon source	Rosfarizan and Ariff (1998)
Optimization of pH control strategy for kojic acid production by *A. flavus* Link 44-1	Rosfarizan *et al*. (2000)
Optimization of cultural conditions of kojic acid production by *Aspergillus parasiticus*	El-Aasar (2006)
Depigmenting effect of kojic acid esters in Hyperpigmented B16F1 Melanoma Cells	Lajis *et al*. (2012)

2.6 KA production via SSF

Interest in SSF has been increasing because of its important applications in producing enzymes, biopesticides, aroma-compounds, biopharmaceuticals, organic acids and other bioactive compounds. Until recently, the feasibility of SSF as renewed mean for kojic acid production was reported by several researchers. Rusley (2012) reported the production of kojic acid from pineapple waste under SSF by *A. flavus* Link 44-1. In the same work, optimal levels of initial moisture content, initial culture pH and incubation time were also identified. In another work by Spencer *et al*. (2012), usability of sago hampas as substrate for kojic acid production was tested and it was revealed that maximum yield of kojic acid was 2.65g per 10g of sago hampas.

In comparison with SmF, SSF in general, offers tremendous economical and environmental advantages in many aspects. The comparison between SmF and SSF is outlined in **Table 2**.

Table 2: Comparison between SmF and SSF (Raimboult, 1998)

Factor	**SmF**	**SSF**
Substrates	Soluble substrates (sugars).	Polymer insoluble substrates: (Starch, Cellulose, Pectines and Lignin)
Aseptic conditions	Heat sterilization and aseptic control.	Vapor treatment and non-sterile conditions.
Water	High volumes of water consumed.	Limited consumption of water.
pH control	Easy pH control.	Buffered solid substrates.
Inoculation	Easy inoculation, continuous process.	Spore inoculation and batch.
Contamination	Risks of contamination for single strain bacteria.	Risk of contamination for low rate growth of fungi.
Energy Requirement	High energy consuming.	Low energy consuming.
Volume of Equipment	High volumes and high cost technology	Low volumes &low costs of equipments
Effluent & pollution	High volumes of polluting effluents	No effluents and less pollution

Nowadays, several techniques have been discovered to purify the kojic acid while some are still under development. Purification and separation of kojic acid produced by *A. flavus* had been done by Basappa *et al*. (1970) where chloroform extraction method was employed. In a more recent work by Lajis *et a*l. (2012), repeated crystallization technique was found able to give high purity of kojic acid of about 99.8%.

3.0 MATERIALS AND METHODS

3.1 Pre-treatment of substrate

Pineapple waste was collected from pineapple plantation site in Kota Samarahan, Sarawak. Firstly, the waste was cleaned with running tap water and rinsed with distilled water. The waste was cut down into small pieces before blended. Then, the waste was dried in an oven for 72 hours (3 days) at $69^{O}C$ until a constant weight ass achieved. Next, the dried product was milled and sieved before to be used in kojic acid fermentation.

3.2 Microorganism

The *A. flavus* Link 44-1 was obtained from Universiti Putra Malaysia, UPM. The strain was sub-cultured on the Potato Dextrose Agar (PDA) and was grown for 7 days at $30^{O}C$. Inoculum was prepared in the form of spore suspension by harvesting using 0.001% (v/v) Tween 80 solution with standardized concentration of 1×10^{6} spore/ml (Rosfarizan, 2000)

3.3 Kojic acid fermentation

Five gram of pineapple waste was used as substrate in each culture. The culture was inoculated with spore suspension 1×10^{6} spore/ml. The cultivation was carried out at $30^{O}C$ at static condition. The optimal level of initial moisture content (70% (v/w)) and pH 3.5 for production of kojic acid from pineapple waste via SSF as stated by Rusley (2012) was applied in this work.

3.4 Extraction

Fifty milliliter of distilled water was added into the sampled culture from SSF experiment. Then the slurry suspension was centrifuged at 6000 rpm for 20 minutes at $4^{O}C$ (Conti *et al*., 2001) and filtered using 0.45 um filter. After that, the slurry suspension was re-filtered through 0.45 um filter pump.

3.5 Assays

3.5.1 Reducing sugar analysis

The Dinitrosalicyclic acid (DNS) method (Miller, 1959) was employed for reducing sugar determination. The absorbance was translated into glucose equivalent using sugar standard graph. 1 ml of supernatant was added to 1 ml of DNS reagent. The DNS reagent was prepared by dissolving 1 g of 3,5- dinitrosalicylic acid, 0.2 g phenol, 0.5 g sodium sulphite and 1 g of NaOH in 100 ml distilled water. Then the mixture was boiled with hot water for about 10 minutes and cooled down. The mixture was added with 1 ml of Roschell salt and the absorbance was read at 575 nm using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan).

3.5.2 Kojic acid analysis

The colorimetry method (Bentley, 1957) was used to quantify kojic acid where 1 ml of diluted sample was mixed with 1 ml of ferric chloride ($FeCl_3$) solution. $FeCl_3$ was prepared by dissolving 1 g of $FeCl_{3.}6H_2O$ in 100 ml of 0.1 N HCl. The reaction between the functional group of hydroxyl and phenolic in the samples produced reddish mixture. The absorbance of the mixture was measured using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan) at wavelength of 500 nm. The kojic acid equivalent was determined from kojic acid standard curve.

3.6 Purification and separation of kojic acid

Kojic acid produced was further purified in order to separate the kojic acid from impurities. The purification method was based on repeated crystallization technique as suggested by Lajis *et al*. (2012). Upon extraction, the sample was centrifuged 6000 rpm. Following that the sample was filtered in order to remove the biomass and other solid particles from the suspension. The sample was dried in rotary evaporator by removing excess water to obtain concentrated sample before it was desiccated in oven for 24 hours for crystallization process.

3.6.1 Characterization of kojic acid

The purified kojic acid was characterized using NMR, FTIR, HPLC and UV spectroscopy. All the NMR spectra were recorded in a JEOL 500 MHz FT-NMR spectrometer. ^{1}H NMR chemical shift was reported relative to TMS as the internal standard and using DMSO-d_6 as the solvent (DMSO-d_6: 2.50 ppm). All the FTIR spectra were recorded as KBr disc using Perkin Elmer Fourier Transform Infrared (FTIR) Spectrum GX1 v5.0 spectrophotometer. Meanwhile, for high performance liquid chromatography (HPLC) reading, Shimadzu Chromatography System (Shimadzu Kyoto, Japan) equipped with Shimadzu LG-20AT was used. UV absorption spectra were recorded using Perkin Elmer Lambda 25 UV-Vis spectrophotometer.

4.0 RESULTS AND DISCUSSION

4.1 Production of kojic acid from pineapple waste by *A. flavus* Link 44-1 via SSF

Figure 1 depicts the profile of reducing sugar and kojic acid over incubation time attained at initial moisture content 70% (v/w) and pH 3.5 with the application of inoculum at 10^6 spore/ml.

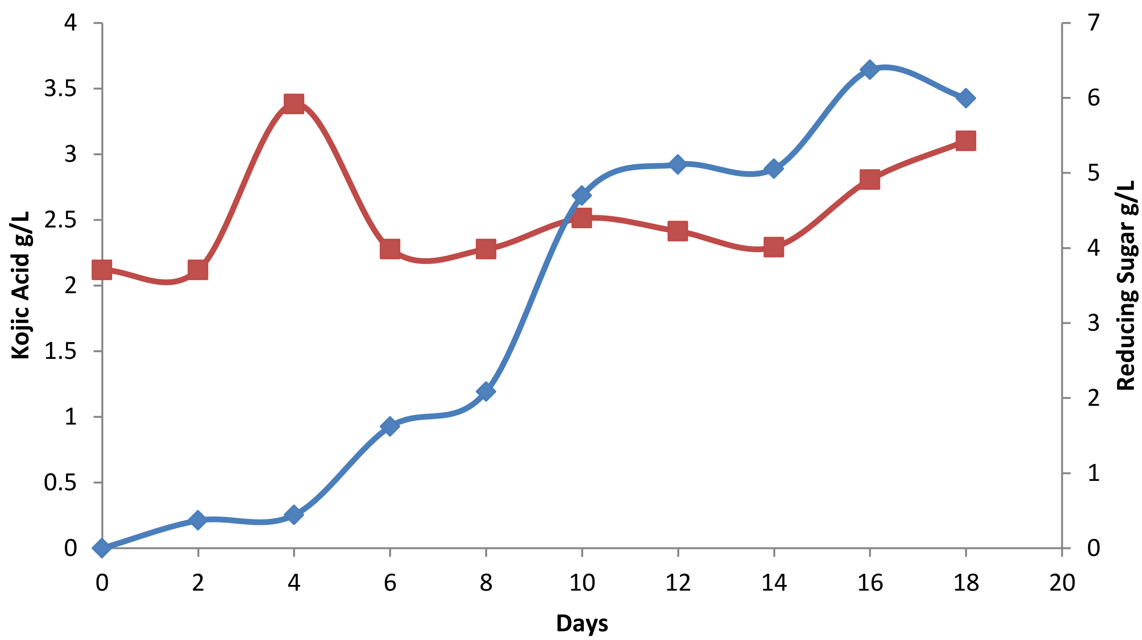

Figure 1: Time course of kojic acid production by *A.flavus* Link 44-1 from pineapple residues at 70% (v/w) initial moisture content and pH 3.5. (■) reducing sugar; (♦) kojic acid

It was shown that the maximum production of kojic acid was 6.372 g/L attained on 16th day. From the graph (**Figure 1**), there was a sudden influx and increase of the reducing sugar profile on the day 4^{th} of fermentation than the initial reducing sugar content which was 2.118g/L per 5 gram pineapple waste. The reducing sugar profile also portrays that there was just slight reduction of reducing sugar throughout the course of fermentation. This is due to the presence of several kinds of sugar in the substrate and some of them might not be favourably consumed for synthesizing kojic acid and rather accumulated in the culture broth. It was revealed that pineapple residues generally contain sucrose, glucose, fructose and other nutrients (Sasaki *et al*., 1991; Krueger *et al*., 1992). From Nadzirah *et al.* (2013) study, it was found that sucrose was the major sugar present in the pineapple waste extract. This result is in agreement with Masniza *et al.* (2000) who reported that pineapple contains 12-15% sugar of which two-third or majority is in the form of sucrose and the rest are glucose and fructose. This circumstance shed some ideas on the importance of effective strategies for making the residual sugars in pineapple waste usable for synthesizing kojic acid in future.

4.2 Purification of kojic acid produced from pineapple waste by *A. flavus* Link 44-1

Figure 2 and **Figure 3** show the formation of concentrated product resulted from kojic acid fermentation using pineapple waste as substrate via SSF. The formation of crystal of kojic acid was rarely seen. Most of the dried particles looked brownish which can be expected as residual sugar. This is further supported by HPLC analysis that indicated 0.025g/L amount of kojic acid from 2.118g/L of initial reducing sugar content and 1.5% (w/w) from NMR analysis per 5 gram substrate. In other works, samples from kojic acid fermentation that employed glucose via SmF and sago starch via SSF were also dried and purified based on the same repeated crystallization technique. **Figure 4** and **Figure 5** show the formation of crystals obtained from both fermentations. Formation of crystal from fermentation of pineapple using *A. flavus* NH9 shows similar results with the results of the experiment using *A. flavus* Link 44-1.

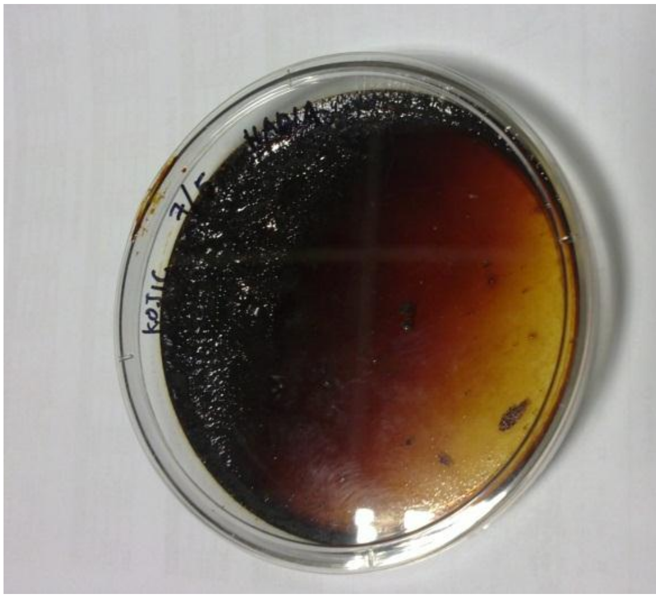

Figure 2: Formation of concentrated product from kojic acid fermentation by *A. flavus* NH9 via SSF.

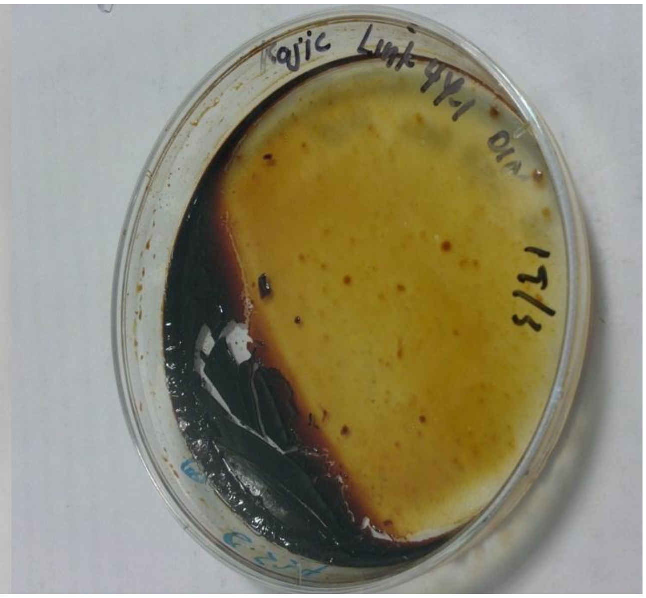

Figure 3: Formation of concentrated product from kojic acid fermentation by *A. flavus* Link 44-1 via SSF.

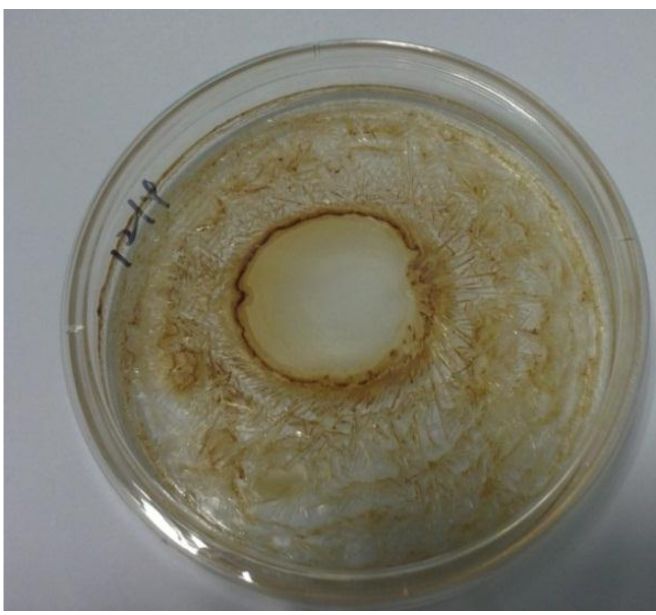

Figure 4: Formation of crystal from kojic acid fermentation by *A. flavus* NH9 using glucose as substrate via SmF.

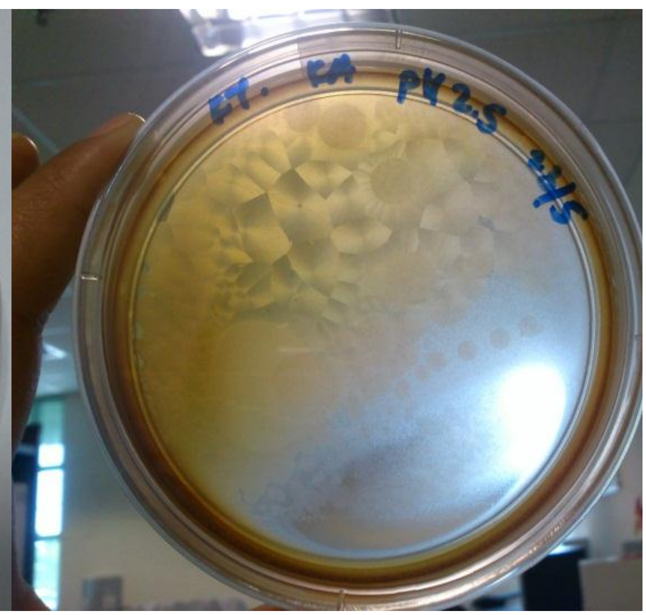

Figure 5: Formation of crystal from kojic acid fermentation by *A. flavus* NH9 using sago starch as substrate via SSF.

It was observed that crystals formed were more visible and the colour less brownish in glucose and sago starch than from pineapple waste. This is due to the present of reducing sugars other than glucose in pineapple waste such as sucrose, fructose and other nutrients (Sasaki *et al*., 1991; Krueger *et al*., 1992). The visibility of kojic acid in **Figure 4** and **Figure 5** can be related with the fact that glucose is revealed as the most ideal kind of carbon source for kojic acid synthesis (Rosfarizan *et al*., 2010). Glucose is converted to kojic acid without any cleavage on the carbon chain making the conversion process more feasible than any other type of carbon source.

4.3 Spectroscopic Characterization of Kojic Acid

The kojic acid (**Figure 6**) that obtained from the fermentation process under initial moisture content 70 %, pH 3.5 and spore suspension 1 x 10^6 spore/ml was characterized using proton nuclear magnetic resonance (^{1}H NMR), UV-Vis spectroscopy and high performance liquid chromatography (HPLC).

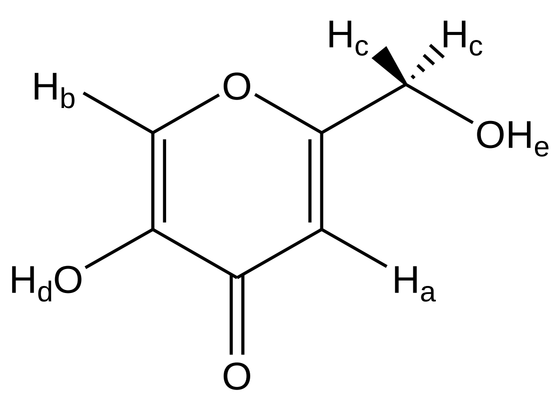

Figure 6: Molecular structure of kojic acid

Figure 7 shows the spectrum of ^{1}H NMR of the kojic acid sample, and it reveals that the kojic acid content in the sample is about ~1.5% w/w, based on the integration of the proton of kojic acid to the proton of water molecule contains in the ^{1}H NMR spectrum. Based on structure of kojic acid (**Figure 6**), the singlets at 7.99 and 6.30 ppm are belongs to the protons (H_a and H_b) that attached to olefin (C=C) carbon, while the singlet located at 4.25 ppm is due to the protons (H_c) from aliphatic carbon CH_2. However, the hydroxyl protons (H_d and H_e) are not obviously observed in the spectrum due to the presence of hydrogen bond with the solvent molecules and also the overlapping with the signal from water. For example, hydroxyl protons H_d should present at around 9.20 ppm, but due to the hydrogen bond formation of this proton with the solvent molecules, this signal becomes a broad signal and is not easily observed especially in the low concentration of sample.