



Faculty of Resource Science and Technology

**PRODUCTION OF KOJIC ACID VIA SOLID STATE  
FERMENTATION (SSF) OF PINEAPPLE WASTE BY  
*Aspergillus flavus* NSH9**

**Nadia Dayana Sikem**

QK  
625  
M7  
N136  
2013

**Bachelor of Science with Honours  
(Resource Biotechnology)  
2013**

# UNIVERSITI MALAYSIA SARAWAK

## THESIS STATUS ENDORSEMENT FORM

**TITLE** PRODUCTION OF KOJIC ACID VIA SOLID STATE FERMENTATION (SSF) OF PINEAPPLE WASTE BY ASPERGILLUS FLAVUS NSH9

**ACADEMIC SESSION:** 2012 / 2013

**I** NADIA DAYANA SIKEM

(CAPITAL LETTERS)

hereby agree that this Thesis\* shall be kept at the Centre for Academic Information Services, Universiti Malaysia Sarawak, subject to the following terms and conditions:

1. The Thesis is solely owned by Universiti Malaysia Sarawak
2. The Centre for Academic Information Services is given full rights to produce copies for educational purposes only
3. The Centre for Academic Information Service is given full rights to do digitization in order to develop local content database
4. The Centre for Academic Information Services is given full rights to produce copies of this Thesis as part of its exchange item program between Higher Learning Institutions [or for the purpose of interlibrary loan between HLI]
5. \*\* Please tick (✓)

☐

CONFIDENTIAL

(Contains classified information bounded by the OFFICIAL SECRETS ACT 1972)

☐

RESTRICTED

(Contains restricted information as dictated by the body or organization where the research was conducted)

☒

UNRESTRICTED

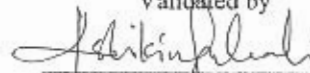
(AUTHOR'S SIGNATURE)

Permanent Address

CAWANGAN TATATERTIB, IPK, SABAH,  
88560, KOTA KINABALU, SABAH

Date: 09/07/2013

Validated by



(SUPERVISOR'S SIGNATURE)

**NURASHIKIN SUHAILI**

Lecturer

Faculty of Resource Science & Technology  
Universiti Malaysia Sarawak  
94300 Kota Samarahan, Sarawak.

Date: 9/7/2013

Notes: \* Thesis refers to PhD, Master and Bachelor Degree  
\*\* For Confidential or Restricted materials, please attach relevant documents from relevant organizations/ authorities

**Production of Kojic Acid via Solid State Fermentation (SSF) of Pineapple Waste by  
*Aspergillus flavus* NSH9**

**Nadia Dayana Sikem**

P.KHIDMAT MAKLUMAT AKADEMIK  
UNIMAS



1000246646

A progress submitted in partial fulfilment of the degree of Bachelor of Science with

Honours

(Resource Biotechnology)

**Supervisor: Miss Nurashikin binti Suhaili**

**Co-supervisor: Assoc. Prof. Dr. Awang Ahmad Sallehin bin Awang Husaini**

Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2013

## ACKNOWLEDGEMENT

I would like to take this opportunity to express my profound gratitude and deep regards to my supervisor, Miss Nurashikin binti Suhaili and my co-supervisor Assoc. Prof Awang Ahmad Sallehin Bin Awang Husaini for their exemplary guidance, monitoring and constant encouragement throughout the course of this thesis. The blessing, help and guidance given by them time to time shall carry me a long way in the journey of life on which I am about to embark.

Furthermore, I would also like to acknowledge with much appreciation of the crucial role of all the lecturers and staff members of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, who gave the permission to use all required equipment and the necessary materials to complete the my research project. I am obliged to the entire master student, for the valuable information and cooperation during the period of my final year project. Special thanks go to my teammate who help me to assemble the parts and gave suggestion upon completion of my thesis.

My deepest gratitude goes to my parent, Mr Sikem ak Chelaha, Mrs Momie binti Gusti and also to my brothers, sisters and friends who has always been there for me, the constant encouragement they give to keep me going and their love to empower me that never fails all the time without which this project would not be possible.

Lastly, I dedicate my utmost gratitude to all individuals that have been indirectly contributed in this research. Your kindness means a lot to me. Thank you very much.

## DECLARATION

I, Nadia Dayana Sikem declare that this thesis is my own work and effort that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature: ..... 

Date: ..... 09 / 07 / 2013

## TABLE OF CONTENTS

Acknowledgement.....	I
Declaration.....	II
Table of content .....	III
List of Abbreviations.....	IV
List of Tables.....	V
List of Figures.....	VI
Abstract.....	1
1.0 Introduction.....	2
2.0 Literature Review.....	5
2.1 Pineapple waste.....	5
2.2 Solid state fermentation.....	7
2.3 Kojic acid.....	9
2.4 <i>Aspergillus flavus</i> NSH9.....	10
3.0 Materials and Methods.....	12
3.1 Pre-treatment of substrate.....	12
3.2 Microorganism.....	12
3.3 Solid state fermentation.....	13
3.3.1 Effect of initial moisture content .....	13
3.3.2 Effect of pH.....	13
3.3.3 Effect of time of incubation .....	14
3.4 Extraction.....	14
3.5 Reducing sugar analysis.....	14
3.6 Kojic acid analysis.....	15
4.0 Results and Discussion.....	16
4.1 Effect of initial moisture content.....	16
4.2 Effect of pH.....	19
4.3 Effect of time of incubation.....	23
4.4 Qualitative analysis of kojic acid.....	25
5.0 Conclusion and Future Recommendation.....	29
6.0 References.....	31
7.0 Appendices.....	35

## LIST OF ABBREVIATION

PDA	Potato dextrose agar
DNS	Dinitrosalicylic acid
SSF	Solid state fermentation
mL	Milliliter
M	Molarity
°C	Degree of celsius
µm	Micrometer
HCL	Hydrochloric acid
g	Gram
H <sub>2</sub> O	Water
nm	Nanometer
g/L	Gram per liter
v/w	Volume per weight

## LIST OF TABLE

### Tables

Table 1	Application of pineapple waste in various bioprocesses.....	6
Table 2	Kojic acid production via SmF from different substrates and microorganisms.....	8
Table 3	Characteristics of <i>A. flavus</i> .....	11
Table 4	Comparison of kojic acid concentration from pineapple waste using colorimetry method and HPLC at different pH levels.....	25



## LIST OF FIGURE

### Figures

<b>Figure 1</b>	<i>A. flavus</i> NSH9 grown on PDA agar.....	10
<b>Figure 2</b>	7-day old harvested <i>A. flavus</i> NSH9 in Tween 80.....	12
<b>Figure 3</b>	Effect of initial moisture content on reducing sugar consumption by <i>A. flavus</i> NSH9 via SSF of pineapple waste.....	17
<b>Figure 4</b>	Effect of initial moisture content on kojic acid production by <i>A. flavus</i> NSH9 via SSF of pineapple waste.....	17
<b>Figure 5</b>	Comparison of maximum production of kojic acid from pineapple waste by <i>A. flavus</i> NSH9 via SSF at different initial moisture content levels.....	19
<b>Figure 6</b>	Effect of pH on reducing sugar consumption by <i>A. flavus</i> NSH9 via SSF of pineapple waste.....	20
<b>Figure 7</b>	Effect of pH on kojic acid production by <i>A. flavus</i> NSH9 via SSF of pineapple waste.....	21
<b>Figure 8</b>	Comparison of maximum production of kojic acid from pineapple waste by <i>A. flavus</i> NSH9 via SSF at different pH levels.....	22
<b>Figure 9</b>	Time course of kojic acid production by <i>A. flavus</i> NSH9 via SSF of pineapple waste at pH 3 and 80% (v/w) initial moisture content.....	24
<b>Figure 10</b>	Concentration of kojic acid analysis using HPLC method at pH 2.5 and 80% (v/w) initial moisture content.....	26
<b>Figure 11</b>	Concentration of kojic acid analysis using HPLC method at pH 3.0 and 80% (v/w) initial moisture content.....	26
<b>Figure 12</b>	Concentration of kojic acid analysis using HPLC method at pH 3.5 and 80% (v/w) initial moisture content.....	27
<b>Figure 13</b>	Concentration of kojic acid analysis using HPLC method at pH 4.0 and 80% (v/w) initial moisture content.....	27
<b>Figure 14:</b>	Concentration of kojic acid analysis using HPLC method at pH 4.5 and 80% (v/w) initial moisture content.....	28

# Production of Kojic Acid via Solid State Fermentation (SSF) of Pineapple Waste by *Aspergillus flavus* NSH9

Nadia Dayana Sikem

Resource Biotechnology Programme  
Faculty of Science and Technology  
Universiti Malaysia Sarawak

## ABSTRACT

Kojic acid is an organic acid that has high commercial values in various industries such as cosmetic, medical, agriculture, food, and chemistry. In this work, we reported the feasibility of pineapple waste for producing kojic acid via solid state bioconversion. The effects of initial moisture content, pH, and incubation time on the cultivation were investigated. The best initial moisture content for kojic acid production via SSF was obtained at 80% (v/w). Maximum production of kojic acid was also notable at pH 3.0 while the optimal range of incubation time for kojic acid production was determined between 10 and 18 days of incubation. The present study proved the promising applicability of pineapple waste as alternative substrate for the production of kojic acid by *Aspergillus flavus* NSH9 via SSF.

Keywords: Pineapple waste, Kojic acid, *Aspergillus flavus* NSH9, Solid state fermentation (SSF)

## ABSTRAK

Asid kojik adalah asid organik yang mempunyai nilai komersial yang tinggi dalam pelbagai industri seperti kosmetik, perubatan, agrikultur, makanan, dan kimia. Dalam penyelidikan ini, keberkesanan sisa nanas untuk menghasilkan asid kojik melalui fermentasi substrat pepejal telah dikaji. Kesan kandungan kelembapan awal, pH, dan masa inkubasi telah dikaji. Kandungan kelembapan awal untuk penghasilan asid kojik melalui fermentasi substrat pepejal telah diperolehi pada 80% (v/w). Penghasilan maksimum asid kojik juga dikesan pada pH 3.0 manakala julat optimum masa inkubasi untuk penghasilan asid kojik telah dikenal pasti antara 10 ke 18 hari. Kajian ini telah membuktikan potensi sisa nanas yang memberangsangkan sebagai substrat alternatif untuk penghasilan asid kojik oleh *Aspergillus flavus* NSH9 melalui fermentasi substrat pepejal.

Kata kunci: Sisa nanas, Asid kojik, *Aspergillus flavus* NSH9, Fermentasi substrat pepejal

## 1.0 INTRODUCTION

Pineapple (*Ananas comosus*) is found in almost all the tropical and sub-tropical areas of the world, and it ranks third in production of tropical fruits, behind bananas and citrus (Paull and Duarte, 2011). Nowadays, there are at least 79 countries in the tropics and sub-tropics produce measurable quantities of pineapple (Paull and Duarte, 2011). In Malaysia, pineapple is one of the commercial fruit crops that provide minor contributors to the economic growth. Commonly, pineapples are planted specifically for domestic fresh consumption. The by-products of pineapple consist of basically residual pulp, peels, stem and leaves. The increasing amount of pineapple production has led to the accumulation of massive wastes which is mainly due to human selection and elimination of components unsuitable for human consumption. The current disposal of pineapple waste poses enormous environmental problem due to high content of organic material and suspended solid (Buckle, 1989).

Research have revealed that some agricultural by-products including pineapple waste can be exploited for various bioconversion processes. For example, the waste from pineapple canneries has been used as the substrate for bromelain, organic acids, and ethanol (Larrauri *et al.*, 1997; Nigam, 1999; Dacera *et al.*, 2009). Nowadays, agro-industrial waste and by-product like sugar cane bagasse (Silva *et al.*, 2002), orange peel (Martin *et al.*, 2000), and other food processing waste (Zheng *et al.*, 2000) are frequently used as alternative substrates for enzyme production or other products either via solid state fermentation (SSF) or submerged fermentation (SmF). This is due to the high amount of the residual compounds in such wastes particularly sugars, minerals, proteins and lignocellulosic materials (Solange *et al.*, 2012). Bio-products can be produced depending

on the microorganism used such as fungi and bacteria (Rosfarizan *et al.*, 2000). In previous research work by Karcem *et al.*, (2010), pineapple waste was used to cultivate *Aspergillus niger* via SSF and it was proven that citric acid was successfully produced from the cultivation. Other works reported include the use of pineapple waste as medium for production of ethanol by *Zymomonas mobilis* (Tanaka *et al.*, 1999) and lactic acid by *Lactobacillus delbruackii* (Abdullah *et al.*, 1998).

Among other bio-products that can be potentially produced from sugary wastes such as pineapple residue via SSF is kojic acid. Kojic acid is an organic acid, which is chemically known as 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one (Nandan and Polasa, 1985) or 5-hydroxy-2-hydroxymethyl-4-pyrone (Kahn *et al.*, 1995). This organic acid has high commercial values in various industries such as cosmetic, medical, agriculture, food, and chemistry. Kojic acid is mainly used in cosmetic industry as skin whitening ingredient. It replaces the role of hydroquinone that has been banned by Food Drug Authority (FDA) some years ago. This has resulted in high demand of kojic acid persistently across the globe. Kojic acid is highly produced by *Aspergillus* spp. and *Penicillium* spp. but *A. flavus* always the chosen and more preferable microorganism due to its ability to produce high amount of kojic acid (Rosfarizan *et al.*, 2000).

To date, little is still known on the use of pineapple waste as substrate for kojic acid production. Thus, the emphasis of this research project was to study the production of kojic acid by *A. flavus* NSH9 using pineapple waste as substrate via SSF. The objectives of this study were:

- i. To determine the usability of pineapple waste as a substrate for kojic acid production by *A. flavus* NSH9 via SSF
- ii. To identify the optimum conditions for kojic acid production by *A. flavus* NSH9 via SSF

## 2.0 LITERATURE REVIEW

### 2.1 Pineapple waste

Pineapple is a collective fruit and native to the Asian tropics, it develops from a whole inflorescence with many flowers and not from a single flower as for example in durians (Chin & Yong, 1992). In general, pineapples are planted specifically for domestic fresh consumption. For example, the cut pieces are used as a dessert, in fruit cocktail mixes, and in salad and cooked meat dishes. Tropical and subtropical fruit processing have considerably higher ratios of by-products than the temperate fruits (Schieber *et al.*, 2001). The by-products of pineapple consist of basically residual pulp, peels, stem and leaves. The increasing amount of pineapple production and the demand in its processing industry has led to the accumulation of massive residues throughout the country. This results in serious environmental pollution since the proper disposal of the waste is expensive due to the high costs of transportation and limited availability of landfills. Apart from that, report have shown that 40-80% of pineapple fruit that are discarded as waste have high amount of organic materials and suspended solid (Bankoffi and Han, 1990). This in turn may cause difficulties in the disposal process due to high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) level in the residues as well as and severe pH condition (Upadhyay *et al.*, 2010).

Research has revealed that agricultural waste including pineapple residues can be potentially used for various bioconversion processes due to notable amount of its beneficial residual compounds such as sugar, starch, and other nutrients (Rani *et al.*, 2004).

Furthermore, production of value-added bio-products from renewable sources via economical SSF process serves many advantages, as it is more cost effective and ecological friendly. Previous researchers have reported several works on the use of pineapple waste as substrate for bioconversion. The details are as outlined in **Table 1**.

**Table 1:** Application of pineapple waste in various bioprocesses

Product	Microorganism	Description	Reference
Bioprotein (function as protein supplementation for human and animal)	<i>Phanerochaete chrysosporium</i> PC-13 (basidiomycetes)	An increase of 0.2% in bioprotein production was achieved on the fifth day of fermentation, after using statistical techniques for media optimization. A higher yield of bioprotein production by liquid state bioconversion using lower concentration (%w/v) of media components (PSS- 1.0, $\text{KH}_2\text{PO}_4$ - 0.10, and $\text{NH}_4 \text{H}_2\text{PO}_4$ - 0.25)	Jamal <i>et al.</i> , (2009)
Citric acid	<i>Aspergillus niger</i>	Maximum citric acid of 60.0 g/kg of pineapple waste was obtained under optimum conditions.	Kareem <i>et al.</i> , (2010)
Ethanol	<i>Zymomonas mobilis</i>	The ethanol yield from pineapple waste was 92% of the theoretical yield and the productivity was $2.81 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ .	Tanaka <i>et al.</i> , (1999)
Lactic acid	<i>Lactobacillus delbrueckii</i>	The highest yield was 85.65 % achieved at $40^\circ \text{C}$ , pH of 6.00, and 52.5 g/l sugar concentration with 5 g/l yeast extract.	Abdullah (2010)
Phenolic antioxidants	<i>Rhizopus oligosporus</i>	Total phenolic content (TPC) of both index were increased, index 1 reached 3.45 mg GAE / g and index 2 reached 3.83 mg GAE/ g after 12 day of growth.	Masimur (2011)

## 2.2 Solid state fermentation (SSF)

SSF is a fermentation process which involves the utilization of water-insoluble substrates for microbial growth and it is usually carried out in solid or semi-solid systems in the near absence of water; however, the substrate must contain sufficient moisture for the growth and metabolism of micro-organism (Pandey, 2003). SSF offers opportunities in processing agro-industrial residues due to the lower energy requirement, higher product yields and productivities, lower capital and operating costs, and it is more environmental-friendly (Solange *et al.*, 2012).

The appropriate selection of substrate is one of the important features in SSF. Solid material acts as physical support and source of nutrients for the cultivation. In contrast with submerged fermentation (SmF), SSF possesses similar or higher yields than those obtained in the SmF, higher end-concentration of products and stability, lower catabolic repression and lower demand on sterility due to low water level used, and culture media are often quite simple because the substrate usually provides all the nutrients necessary for growth. (Vidayalakshmi, 2009). The low water volume used in SSF has a very large impact on the economy of the process mainly because of the smaller fermenter-size, the reduced downstream processing, the reduced stirring and lower sterilization costs (Hölker & Lenz, 2005; Nigam, 2009).

In previous works, SmF is widely used for production of kojic acid from various types of feedstock and by several different *Aspergillus* strains. **Table 2** outlined several works related as reported in the literature.



**Table 2:** Kojic acid production via SmF using different substrates and microorganisms

Substrate	Microorganism	Description	Reference
Gelatinized and hydrolyzed sago starch	<i>Aspergillus flavus</i>	Kojic acid production (23.5 g/L) using 100 g/L sago starch in a shake flask was comparable to fermentation of glucose (31.5 g/L) and glucose hydrolyzate (27.9 g/L) but in the 50-L fermentor was greatly reduced due to non-optimal aeration conditions. Kojic acid production using glucose was higher in the 50-L fermentor than in the shake flask.	Rosfarizan <i>et al.</i> (1998)
Partially hydrolyzed raw corn starch	<i>Aspergillus oryzae</i> MK-107-39	In the cultivation in the airlift bioreactor using SM1, nearly 40g/l of kojic acid was produced, which was the same as the amount produced in the jar fermenter containing GM1.	Futamura <i>et al.</i> (2001)
Glucose and Polypepton	<i>Aspergillus oryzae</i>	A production rate of 4.44 g/l·d was obtained when the initial glucose and polypepton concentrations were 148.0 g/l and 4.8 g/l, respectively, at a $k_1$ value of 164.7 h <sup>-1</sup> .	Takamizawa <i>et al.</i> (1996)
Sucrose	<i>Aspergillus candidus</i>	0.5 g kojic acid/g sucrose was yield in the fermentation using sucrose that produced about two times higher than the yield obtained from glucose, that is, 0.25 g kojic acid/g glucose.	Wei <i>et al.</i> (1991)
Glucose	<i>Aspergillus parasiticus</i>	The highest level of kojic acid (34.38 g L <sup>-1</sup> ) was obtained by <i>A. parasiticus</i> using fermentation medium of 6% glucose, 1% yeast extract with initial pH 5 and incubated at 28°C for 10 days under rotary shaking culture (220 rpm).	El-Aasar (2006)

Currently, there are few works that initiate the use of agricultural waste for kojic acid via SSF. To the best of our knowledge, no work has yet been reported on the use of pineapple waste as feedstock for the production of kojic acid.

### 2.3 Kojic acid

Kojic acid is an organic acid, which is biologically produced by different types of fungi in aerobic fermentation using various substrates (Rosfarizan *et al.*, 2010). This organic substance can be found in leftover residues from fermenting natural foods for example, rice, soy, wheat and pineapple (Sardjono, 1998). It is used mostly in cosmetic as whitening agent and as UV light protector for many decades (Kobayoshi *et al.*, 1996). In agriculture, kojic acid functions as an anti-melanosis by inhibiting polyphenol oxidase during post-harvest of product. Besides that, kojic acid also serves as an important compound for other industries such as in medical as antibacterial and pain killer (Nohynek *et al.*, 2004) and also in food processing as an antioxidant and flavour enhancer (Burdock *et al.*, 2001).

Kojic acid fermentation can be divided into two phases, which are growth phase and production phase. During the growth phase, relevant enzymes in kojic acid metabolic pathway that are responsible for the degradation of starch into fermentable sugar are secreted while kojic acid is synthesized during the secondary phase (Rosfarizan *et al.*, 2002). It is revealed that kojic acid is produced by direct conversion from glucose throughout multistep reaction without any cleavage into small fragments (Bently, 1957). Nonetheless, some studies have proved that kojic acid could also be derived from several kinds of carbon sources such as corn starch (Futamura *et al.*, 2001), sago and potato starch (Rosfarizan *et al.*, 1998) and sucrose (Rosfarizan *et al.*, 2007). This further implied the usability of starchy and sugary materials as substrate for kojic acid production.

## 2.4 *Aspergillus flavus* NSH9

*Aspergillus* is a filamentous and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. *Aspergillus* is saprophytes, whereby it obtains its nutrition from dead organic matter. *A. flavus* is one of the common species of *Aspergillus*. The color of the *A. flavus* colony on surface surface, as shown in **Figure 1**, is yellowish to greenish or brown mold with a goldish to red brown in colour (Hedayati *et al.*, 2007). **Table 3** outlines some basic features of *A. flavus*.



**Figure 1:** *A. flavus* NSH9 grown on PDA agar

**Table 3:** Characteristics of *A. flavus* (Hedayati *et al.*, 2007).

Criteria	Characteristic
Microscopic morphology	Length usually less than 1 mm, heavy walled, uncolored, and coarsely roughened
Macroscopic morphology of vesicles	Elongated when young, later becoming sub-globose or globose (round, radiate head), varying from 10 to 65 $\mu\text{m}$ in diameter.
Macroscopic morphology of phialides	Biseriate (vesicles produces sterile cells known as metulae that support the conidiogenous phialides) or uniseriate. The primary branches are up to 10 $\mu\text{m}$ , and the secondary branches up to 5 $\mu\text{m}$ .

The ability of *Aspergillus sp.* such as *A. flavus* Link 44-1 (Rosfarizan *et al.*, 1998) to ferment carbohydrate-containing substrate has eased the production of kojic acid and created the advantage of using the low cost agro waste that contains high amount of carbohydrate such as pineapple waste. Previously in research work by Rosfarizan and her co-workers (1998), sago starch was employed in the production of kojic acid by *A. flavus* Link 44-1 via SmF. It was observed that kojic acid production (23.5 g/L) using 100 g/L sago starch in a shake flask was comparable to fermentation of glucose (31.5 g/L) and glucose hydrolyzate (27.9 g/L) but in the 50-L fermentor was greatly reduced due to non-optimal aeration conditions. Kojic acid production using glucose was higher in the 50-L fermentor than in the shake flask.

*A. flavus* NSH9 was isolated from sago humus. The strain has been applied for producing kojic acid from sago hampas via SSF (Spencer *et al.*, 2012) and notable production was successfully attained with the maximum production at 262g/kg hampas after 15 days. At a recovery of 26.5%, this shows the potential of sago hampas, obtained from sago effluent as an alternative substrate for kojic acid production through SSF.

### 3.0 MATERIALS AND METHODS

#### 3.1 Pre-treatment of substrate

Pineapple agriculture waste was collected from fruit stall in Desa Ilmu at Kota Samarahan, Sarawak. Firstly, pineapple waste was cleaned with running tap water and rinsed with distilled water. Then, the pineapple waste was chopped into small pieces before blending. After that, the product was dried in an oven for 72 hours (3 days) at 69°C or until a constant weight was achieved. Lastly, the dry substrate was sieved before being used for fermentation.

#### 3.2 Microorganism

*A. flavus* NSH9 strain was obtained from Department of Molecular Biology, UNIMAS. The strain of *A. flavus* NSH9 was subcultured using Potato Dextrose Agar (PDA) for 7 days at 30°C. Inoculum was prepared in the form of spore suspension with standardized concentration of  $1 \times 10^5$  spore/ml (Rosfarizan *et al.*, 2000).

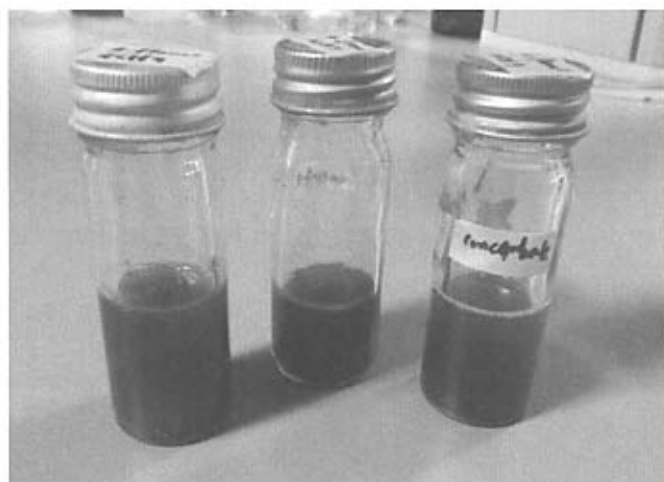


Figure 2: 7-day old harvested *A. flavus* NSH9 in Tween 80.

### 3.3 Solid State Fermentation (SSF)

For the substrate, 5 g of pineapple waste was used in each culture. The culture was inoculated with spore suspension of  $1 \times 10^5$  spore/ml. The cultivation was carried out at room temperature with static condition. The effects of several parameters namely initial moisture content, pH, and incubation time on SSF of pineapple waste by *A. flavus* NSH9 were investigated. The details are mentioned in section 3.3.1 – 3.3.3.

#### 3.3.1 Effect of initial moisture content on SSF of pineapple waste by *A. flavus* NSH9

The amount of initial moisture content of the fermentation was adjusted to 60%, 65%, 70%, 75%, and 80% (v/w) with sterile distilled water prior to incubation. The initial moisture content was determined by considering the volume of inoculum used. The best level of initial moisture content that supports high kojic acid production was applied in further experimental runs.

#### 3.3.2 Effect of pH on SSF of pineapple waste by *A. flavus* NSH9

The effect of pH on kojic acid production from pineapple waste by *A. flavus* NSH9 was determined by testing different levels of initial pH on the cultivation. The initial pH of cultures was adjusted to pH; 2.5, 3.0, 3.5, 4.0, and 4.5. The best pH level that yields the highest production of kojic acid was identified.

### 3.3.3 Effect of time of incubation on SSF of pineapple waste by *A. flavus* NSH9

The effect of incubation time on kojic acid fermentation was studied by setting the cultivation at different period of time. The cultures were aseptically sampled at an interval of 48 hours (2 days), 96 hours (4 days), 144 hours (6 days), 192 hours (8 days), 240 hours (10 days), 288 hours (12 days), 336 hours (14 days), 384 hours (16 days), and 432 hours (18 days). The best range of incubation period where high production of kojic acid occurs was identified.

### 3.4 Extraction

The sampled culture was added with 40 ml of distilled water. Then, the slurry suspension was centrifuged at 6000 rpm for 20 minutes at 4°C (Conti *et al.*, 2001). Next, the suspension was filtered through 0.45 µm filter. The residues were discarded and the supernatant was used in reducing sugar and kojic acid assays. The assays were prepared in duplicate and the results for the assays were expressed as means of duplicate.

### 3.5 Reducing sugar analysis

In this work, dinitrosalicylic acid (DNS) method (Miller, 1959) was used for the reducing sugar determination. The absorbance was translated into glucose equivalent using glucose 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. The mixture was boiled for about 10 minutes and then cooled. Then, the mixture was added with 1 ml of Roschell salt. The



absorbance of the reaction mixture was read at 575 nm by using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan).

### 3.6 Kojic acid analysis

Quantification of kojic acid was carried out using colorimetry method (Bentley, 1957) where 1 ml of diluted sample was mixed with 1 ml of ferric chloride ( $\text{FeCl}_3$ ) solution.  $\text{FeCl}_3$  was prepared by dissolving 1 g of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 100 ml of 0.1 N HCL (Rosfarizan *et al.*, 2000). The reaction between the functional group of hydroxyl and phenolic in the samples produced reddish purple mixture. The absorbance of the reaction mixture was measured by using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan) at a wavelength of 500 nm. The absorbance was translated into kojic acid equivalent based on the standard curve. The colorimetry method was widely used in the kojic acid analysis due to its flexibility compared to other methods. Additionally, the confirmation of kojic acid was performed by high performance liquid chromatography (HPLC) method (Ariff *et al.*, 1996) using a UV detector at 265 nm. The mobile phase constituted a mixture of 50 mM phosphate buffer pH3 and methanol in ratio 95:5 while the stationary phase was a Hibar preppacked column RT 250-4 Lichrosorb RP-18 (10um).