



Faculty of Resource Science and Technology

**SOLID STATE FERMENTATION (SSF) OF SAGO STARCH FOR KOJIC ACID
PRODUCTION**

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Solid State Fermentation (SSF) of Sago Starch for Kojic Acid Production

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This project is submitted in partial fulfilment of the requirement for the Degree of
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Declaration

I hereby declare that the study entitled 'Solid State Fermentation of Sago Starch for Kojic Acid Production' is my original work and that all sources that I have quoted and referred to have been acknowledge by means of complete references. It has been submitted and shall not be submitted in any form to any institution or other university.

(KHATIJAH BINTI BASRI)

Date:

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LIST OF ABBREVIATIONS

Solid State Fermentation	SSF
Submerged Fermentation	SmF
Potato Dextrose Agar	PDA
High Performance Liquid Chromatography	HPLC

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Solid State Fermentation of Sago Starch for Kojic Acid Production

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ABSTRACT

Malaysia produces an abundant supply of sago starch particularly in Sarawak. Owing to its starch content, sago starch can be used to produce kojic acid. Kojic acid is one of the highly demanded organic acids due to its extensive applications in medical, agriculture, food, cosmetic and chemistry. In this work, the potential of *A. flavus* NSH9 in producing kojic acid and the optimum conditions of kojic acid production from sago starch by *A. flavus* NSH9 via SSF was identified. The optimum initial moisture content was found at 60% initial moisture content and the optimum inoculum size for kojic acid production obtained from this study was 10^8 spores/ml. On the other hand, the optimum pH level for maximum kojic acid production from sago starch as sought from this study was pH2.5. Meanwhile, the optimal range of incubation period for kojic acid production from sago starch via SSF was identified between 8 and 18 days. The feasibility of the present research work demonstrated the promising potentiality of sago starch as alternative substrate for kojic acid production by *A. flavus* NSH9 via SSF.

Keywords: *Aspergillus flavus* NSH9, kojic acid, sago starch, solid state fermentation (SSF).

ABSTRAK

Malaysia mengeluarkan satu bekalan berlebihan kanji sago terutama di Sarawak. Disebabkan oleh kandungan kanji tersebut, kanji sago dapat digunakan untuk penghasilan asid kojik. Asid kojik merupakan salah satu asid organik yang mendapat permintaan tinggi disebabkan penggunaan meluasnya dalam bidang perubatan, pertanian, makanan, kosmetik dan kimia. Dalam tugas ini, potensi *A. flavus* NSH9 dalam mengeluarkan asid kojik dan keadaan optimum pengeluaran asid kojik dari kanji sago oleh *A. flavus* NSH9 melalui fermentasi keadaan pepejal telah dikenal pasti. Permulaan optimum kandungan lembapan didapati di 60% kandungan lembapan awal dan optimum inoculum saiz untuk pengeluaran asid kojik diperolehi dari kajian ini ialah 10^8 spora / ml. Sebaliknya, pH optimum untuk maksimum pengeluaran asid kojik dari sago kanji seperti dicari daripada kajian ini ialah pH2.5. Manakala, julat optimum tempoh penderaman untuk pengeluaran asid kojik dari sago kanji melalui fermentasi keadaan pepejal dikenal pasti di antara 8 dan 18 hari. Melalui kerja kajian ini telah menunjukkan penggunaan kanji sago yang menjanjikan sebagai substrat alternatif untuk pengeluaran asid kojik oleh *A. flavus* NSH9 melalui fermentasi keadaan pepejal.

Kata kunci: *Aspergillus flavus* NSH9, asid kojik, kanji sago, fermentasi keadaan pepejal.

1.0 INTRODUCTION

Malaysia produces an abundant supply of sago starch particularly in Sarawak and exported more than 50 965 tonnes in 2011 (DoS, 2012). Sago starch is isolated from sago palm (*Metroxylon spp.*). It is a species from which useful quantities of starch-rich flour can be extracted from the stem tissue by shredding and sedimentation in water (Wina *et al.*, 1986).

The wide availability of sago starch particularly in Sarawak and its functionality has enabled its application in the production of glucose and many different kinds of food such as vermicelli, bread, crackers and biscuits (Karim *et al.*, 2008). Besides that, it is also widely used as the alternative substrate for production of various kinds of bioproduct such as ethanol (Haska and Ohta, 1992; Veera *et al.*, 2006), monosodium glutamate (Zulpilip *et al.*, 1991), cyclodextrin (Solichin, 1995), lactate (Ishizaki, 2002) and glucose syrup (Gorinstein *et al.*, 1994).

Owing to the starch content of the sago starch, one of the potential metabolites that can also be produced is kojic acid, 2-hydroxymethyl-5-hydroxy- γ -pyrone. It is an organic acid produced biologically by different types of fungi during aerobic fermentation using various substrates (Kitada *et al.*, 1967; Ariff *et al.*, 1996; Wakisaka *et al.*, 1998; El-Aasar, 2006). Kojic acid serves as an important metabolite for variety of commercial applications such as in medicine, agriculture, food, cosmetic and chemistry.

Conventionally, kojic acid is produced industrially in aerobic conditions under submerged fermentation (SmF). However, one of the major hindrances in its industrial production is the high cost of raw materials. Utilization of the sago starch which is an inexpensive carbon source, offers the advantage of reducing the cost of raw material for kojic acid production. Furthermore, the feasibility of *A. flavus* NSH9 a local isolate, in producing kojic acid has yet to be investigated. Though production of kojic acid using sago starch via SmF was reported by Rosfarizan *et al.*, (1998) but none was found yet for the production via solid state fermentation (SSF).

The present research project was aimed to study the production of kojic acid from sago starch using *A. flavus* NSH9 via SSF. Therefore the objectives of this study were as follow:

- i. To study the potential of *A. flavus* NSH9 in producing kojic acid from sago starch via SSF.
- ii. To identify the optimum conditions of kojic acid production from sago starch by *A. flavus* NSH9 via SSF.

2.0 LITERATURE REVIEW

2.1 Sago starch

Sago starch is isolated from sago palm (*Metroxylon spp.*) which is widely planted in South East Asia. It is a species from which useful quantities of starch-rich flour can be extracted from the stem tissue by shredding and sedimentation in water (Wina *et al.*, 1986). Sago starch has been used for a long time in the food industry.

It is an important resource especially to the people in rural areas because it has various uses mainly in the production of starch either as sago flour or sago pearl (Mohamed *et al.*, 2008). Besides that, sago flour also serves as staple food by the Melanaus in Sarawak (Sim, 1986). Sago starch is also commonly used in the production of vermicelli, bread, crackers and biscuits (Karim *et al.*, 2008). **Table 1** outlines various applications of sago starch in several industrial fields.

Furthermore, sago starch has also been increasingly applied in various research works producing extensive range of metabolites. Veera *et al.*, (2006) reported that high amount of ethanol 55.3 g/L could be produced from sago starch via submerged fermentation. While, Chittra *et al.*, (2007) have shown that 0.85 g/L of kefiran was produced using sago starch as substrate. In another study by Lim (1991) sago starch has been used to formulate livestock and poultry feed. Based on these studies it is apparent that sago starch can be feasibly used for various bioconversions.

Table 1. Applications of sago starch.

Field	Application	Reference
Food	Thickener, additive and manufacture of noodles	Takahasi (1986); Zulpilip <i>et al.</i> (1991)
	Vermicelli	Lee <i>et al.</i> (2002)
	Japanese confectionary	Hamanishi <i>et al.</i> (2002)
	Glucose syrup	Gorinstein <i>et al.</i> (1994)
	Jellies and pudding	Takahashi (1986); Bujang and Ahmad (2000)
Non-food	Microencapsulation material	Varavinit <i>et al.</i> (2001)
	Biodegradable plastic	Griffin <i>et al.</i> (1997)
	Extender in urea formaldehyde adhesives, finishing agent in production of paper and sizing in textile industry	Sumadiwangsa (1985)
	Component of glue in the plywood and in Glue gel and liquid gel	Bujang <i>et al.</i> (2000)
Biotechnology	Adhesives	Zulpilip (1991)
	Ethanol	Haska and Ohta (1992); Veera <i>et al.</i> (2006)
	Monosodium glutamate	Zulpilip <i>et al.</i> (1991)
	Cyclodextrin	Solichin (1995)
	Lactate	Ishizaki (2002)
Others	Exopolysaccharide kefiran	Chittra <i>et al.</i> (2007)
	Livestock and poultry feed component	Lim (1991)

2.2 Solid state fermentation

Solid state fermentation (SSF) is defined as fermentation involving solids in absence (or near absence) of free water. However, the substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey, 2003). Even though SSF during the previous two decades was termed as a 'low-technology' system, it is now appear as a promising mean for producing extensive range of value- added products such as biopharmaceuticals (Pandey, 2003). The increasing cost of feed for SmF, value addition to agro-industrial residues and ease of operation makes the SSF process more favourable over SmF. SSF process also requires less initial capital and incurs low operating cost and thus suitable for developing agriculture-based economies (Singh *et al*, 2007).

Various fermentation techniques have been developed for producing kojic acid but very few attempts had been made to employ SSF method. To date, employment of SSF for kojic acid production using sago starch have been scarcely reported. Kharchenko (1999) reported that a comparatively high yield of kojic acid (8.5- 9.5 g kojic acid/kg substrate) was obtained in a SSF using grains and grain-forages with high amount of protein and carbohydrates.

Rosfarizan *et al.*, (1998) also reported an amount of 23.5 g/L kojic acid was produced by the cultivation of *A. flavus* Link 44-1 in fermentation of 100 g/L gelatinized starch using shake flask under submerged fermentation (SmF). Little is still know about the feasibility of SSF as a renewed mean for kojic acid production. Thus its employment as a mean production continues to serve as subject of research and studies.

2.3 Kojic acid

Kojic acid is an organic acid, produced biologically by different types of fungi during aerobic fermentation using various substrates (Kitada *et al.*, 1967; Ariff *et al.*, 1996; Wakisaka *et al.*, 1998; El-Aasar, 2006). It is produced by *Aspergillus* spp. and *Penicillium* spp. The *flavus-oryzae-tamarii* groups were reported to have the ability to produce large amounts of kojic acid (Arnstein and Bently, 1956; Kitada *et al.*, 1967; Ariff *et al.*, 1996; Bajpai *et al.*, 1981). On the other hand, Rosfarizan *et al.*, (2010) mentioned that the kojic acid crystallises in the form of colourless, prismatic needles that sublime in vacuum without any changes and the melting point of kojic acid ranges from 151°C - 154°C (Ohyama and Mishima, 1990).

Kojic acid is commonly used in cosmetics and health care industries. It primarily functions as the basic material for the production of skin whitening creams, skin protective lotions, whitening soaps and tooth care product (Rosfarizan *et al.*, 2010). In addition, kojic acid has the ability to suppress hyperpigmentation in human skins by restraining the formation of melanin through the inhibition of tyrosinase, the enzyme that responsible for skin pigmentation (Ohyama and Mishima, 1990; Noh *et al.*, 2009). At present, kojic acid is primarily used as the basic ingredient for excellent skin lightener in cosmetics creams, where it is used to block the formation of pigment by the deep cells on the skins (Masse *et al.*, 2001). As mentioned by Bajpai *et al.*, (1981) glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid. It is formed directly from glucose without any cleavage of the carbon chain into the smaller fragments (Arnstein and Bently, 1956).

Nonetheless, discovery of few kojic acid producing strains that are able to degrade starch to fermentable sugars have sparked some interest to utilize starch- containing materials as the feedstock for kojic acid production. For example, Rosfarizan *et al.*, (1998) have revealed the usability of gelatinized and hydrolysed sago starch as the feedstock for kojic acid by the aids of *A. flavus* Link 44-1 via SmF. In other studies by Spencer *et al.*, (2012), kojic acid could also be produced from sago hampas via SSF using the same strain. The development of kojic acid fermentation is largely dependent on the selection of producing strains, substrates as well as establishment of optimal operating conditions.

2.4 *Aspergillus flavus* NSH9

A. flavus NSH9 provided by Department of Molecular Biology was isolated from sago humus (Mut and Awg Husaini, 2009). *A. flavus* is a filamentous fungus and capable of growing on many nutrient sources. This filamentous fungus was preferred in the production of enzyme both in SmF and SSF. According to Bajpai *et al.*, (1981) *A. flavus* has been reported to have the ability to produce large amount of kojic acid.

Furthermore, it was revealed by Rosfarizan *et al.*, (1998) that *A. flavus* is able to secrete amyolytic enzymes such as α - amylase and glucoamylase during its cultivation which facilitate the degradation of starch easily before kojic acid fermentation takes place. *A. flavus* colonies grow rapidly in hot and dry condition. Colonies are yellowish to green in colour, and consist of a dense felt of conidiospores (CMPT Mycology Plus 0801-3, 2008) as shown in **Figure 1. Table 2** shows some works on the applications of *A. flavus* in producing kojic acid using various substrates.

Table 2. Applications of *A. flavus*.

Microorganism	Substrate	Max yield (g/g)	Reference
<i>A. flavus</i>	Glucose	0.260	Rosfarizan (2000)
	Sucrose	0.240	
	Fructose	0.064	
<i>A. flavus</i> Link 44-1	Sago hampas	0.265	Spencer, <i>et al</i> (2011)
	Sago hampas hydrolyzate	0.059	Nurashikin <i>et al</i> (2012)
	Glucose hydrolyzate from sago hampas	0.279	Rosfarizan <i>et al</i> (1998)
	Gelatinized starch	0.235	
<i>A. flavus</i> S33-2	Potato starch	0.034	Rosfarizan <i>et al</i> (1997)
	Corn starch	0.210	
	Sago starch	0.006	
	Glucose	0.240	



Figure 1: Microscopic morphology of *A. flavus*. Image retrieved from http://www.sciencephoto.com/image/13501/530wm/B2500555-SEM_of_AspERGILLUS_flavus-SPL.jpg

3.0 MATERIALS AND METHODS

3.1 Microorganism

The kojic acid producer, *A. flavus* NSH9 that was isolated from sago humus was obtained from Department of Molecular Biology, Faculty of Resource Science and Technology, University Malaysia Sarawak (UNIMAS). The fungus was subcultured from the stock culture on potato dextrose agar (PDA) media. The fungus was grown at 37°C for 5- 7 days for the development of spores as shown in **Figure 2**. The spore grown on PDA was harvested using appropriate amount of 0.001% (w/v) Tween-80 solution and the spore concentration was determined by using haemocytometer. Inoculum was prepared in the form of spore suspension varied according to the range mentioned in Section 3.3.2.

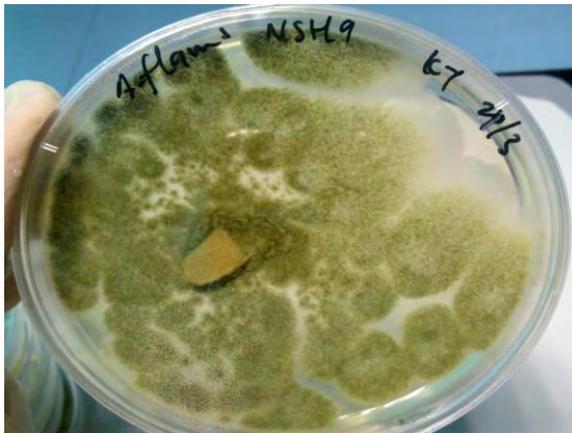


Figure 2: 7-day old *A. flavus* NSH9 on PDA.

3.2 Substrate

Sago flour was utilized as substrate for kojic acid fermentation. The flour was oven-heated at 60°C for 4 days prior to fermentation. In all replicates the gelatinized starch was used as a positive control. Gelatinized starch was prepared by heating the starch slurry to slightly above 70 °C (Rosfarizan *et al.*, 1998).

3.3 Kojic acid fermentation

The production of kojic acid was carried out under SSF. The culture was initiated from 5 gram of sago flour that was placed in petri dish. The 5 gram flour was moistened with 1 ml of salt solution containing 0.05% (w/v) MgSO₄·7H₂O, 0.2% (w/v) KH₂PO₄ and 1% (w/v) filtered urea. The medium was sterilized and cooled before it is inoculated. The cultures were incubated at 37°C in static condition at various initial moisture contents, inoculums size, initial culture pH, and incubation times (as mentioned in Section 3.3.1, 3.3.2, 3.3.3, and 3.3.4 respectively). All experiments were carried out in duplicate. **Figure 3** shows the *A. flavus* NSH9 cultivated on sago starch.

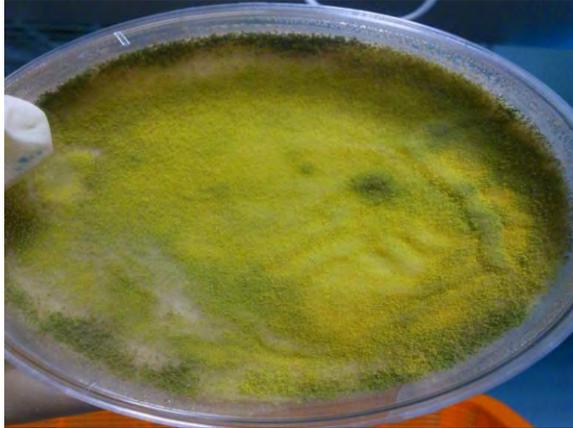


Figure 3: *A. flavus* NSH9 cultivated on sago starch after 14 days of incubation at 60% (v/w) initial moisture content.

3.3.1 Effects of initial moisture content

Optimization of initial moisture content on kojic acid fermentation was carried out by incubating the cultures at 60%, 70%, 80% (v/w). The moisture content was adjusted by considering total volume that includes the distilled water, mineral salt solution and inoculum suspension. The optimum initial moisture content attained was applied for subsequent experimental runs.

3.3.2 Effects of inoculum size

Optimization of inoculum size was carried out by varying the spore concentration at 10^5 , 10^6 , 10^7 , 10^8 spores/ ml. The optimum level of spore concentration attained was then applied for subsequent fermentations.

3.3.3 Effects of pH

Optimization of initial pH was carried out by adjusting the initial pH of the culture at pH 2.5, 3.0, 3.5, 4.0. The pH of the medium was adjusted using 1 M HCl or 1 M NaOH. The optimum level of pH obtained was maintained for the following experimental runs.

3.3.4 Effects of incubation time

The effect of incubation time on kojic acid fermentation was determined by sampling the cultures at 2 days interval for 20 days while maintaining other parameters (initial moisture content, inoculums size, and pH) at their optimal levels.

3.4 Extraction

The extraction of the samples was done by adding 30 mL of distilled water to the sampled cultures. The slurry suspension was mixed thoroughly. Then, the slurry suspension was centrifuged at 6000 rpm at 4°C for 20 minutes (Conti *et al.*, 2001). The supernatant was filtered through 0.45 µm filter prior to reducing sugar and kojic acid assays. The assays were prepared in duplicate and the result will be expressed as means of duplicate values.

3.5 Analytical methods

Samples were aseptically withdrawn for every 2 days. The sampled cultures were analysed for the following tests.

3.5.1 Reducing sugar

The dinitrosalicylic acid (DNS) method (Miller, 1959) was employed for the reducing sugar analysis where 1 ml of supernatant was added with 1 ml of DNS reagent. The

reagent can be 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. After that, the mixture was boiled with hot water for about 10 minutes and then cooled. Then, the mixture were added with 1 mL of Roschell salt and the absorbance of the mixture was measured at 575 nm by using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan) and then translated into glucose equivalent based on glucose standard graph.

3.5.2 Kojic acid

Kojic acid was quantified using colorimetry method (Bentley, 1957) where 1 ml of diluted sample was mixed with 1 ml of ferric chloride (FeCl_3) solution. FeCl_3 solution can be prepared by dissolving 1 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 ml of 0.1 M HCl. The absorbance of the reaction mixture was measured using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan) at a wavelength of 500 nm. The confirmation of kojic acid was also based on the high performance liquid chromatography (HPLC) method by Ariff *et al.*, (1996). HPLC method used was under UV detector at 260 nm. The mobile phase constitutes of a mixture of 50 mM phosphate buffer pH3 and methanol in ratio 95:5 while the stationary phase is a Hibar prepacked column RT 250-4 Lichrosorb RP-18 (10 μm).

3.6 Statistical analysis

Statistical analysis was done by using Analyse-it software (version 2.26 Analyse-it, Inc., Leeds, UK). The mean values were compared by applying Tukey method via One-Way Analysis of Variance (ANOVA).

4.0 RESULTS AND DISCUSSION

4.1 Effect of initial moisture content on SSF of sago starch by *A. flavus* NSH9

In this study, the initial moisture content of the cultures was adjusted at 60%, 70%, and 80% (v/w). The profiles of reducing sugar consumption and kojic acid production are illustrated in **Figure 4- 7** In general, as shown by **Figure 4** and **Figure 6**, the reducing sugar was decreased after 10th and 6th day for sago starch and gelatinized starch cultures respectively. The highest kojic acid production was found at 60% (v/w) initial moisture content with the concentration of 4.455 g/L attained on day 16 in fermentation that employed sago starch while in gelatinized starch cultures, the maximum production was 7.8 g/L obtained on 14th day of incubation.

The higher production of kojic acid in gelatinized starch cultures compared to sago starch cultures is understood due to direct conversion of sugar resulting from starch that has been readily gelatinized to kojic acid. In contrast, in sago starch cultures, the biosynthesis of kojic acid occurred in two stages whereby the starch was first degraded by the amylolytic enzymes such as amylase and glucoamylase (Rosfarizan *et al.*, 1998) before the resulting simpler sugars were converted to kojic acid.

As depicted by **Figure 5**, the production of kojic acid from sago starch was apparently reduced when the initial moisture content was increased beyond 60% (v/w). In contrast there was only slight difference in terms of the profile of kojic acid production from gelatinized starch when the initial moisture content was varied from 60% to 80% (v/w) as shown in **Figure 7**.