



Faculty of Resource Science and Technology

**SOLID SUBSTRATE FERMENTATION (SSF) OF PINEAPPLE WASTE
USING *ASPERGILLUS FLAVUS* LINK 44-1**

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LIST OF ABBREVIATIONS

PDA	Potato Dextrose Agar
SSF	Solid Substrate Fermentation
mL	Milliliter
M	Molarity
°C	Degree of celcius
µm	Micrometer
HCl	Hydrochloric acid
g	Gram
H²O	water
nm	Nanometer
g/L	Gram per Liter
v/w	Volume per weight

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Solid Substrate Fermentation (SSF) of pineapple waste by *Aspergillus flavus* Link 44-1

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ABSTRACT

A solid state fermentation of pineapple waste by *Aspergillus flavus* Link 44-1 was developed for kojic acid production. Kojic acid is an organic acid that is widely used as an ingredient for dermatological products, precursor for flavor enhancer and also as anti-inflammatory drug. In this work, 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 70% (v/w). Maximum production of kojic acid was also attained at pH 3.5 while the optimal range of incubation time for high kojic acid production was seen between 8 and 16 days of incubation. The present study proved the feasibility of pineapple waste as alternative substrate for kojic acid production by *A. flavus* Link 44-1.

Key words: Kojic acid, *Aspergillus flavus* Link 44-1, Solid Substrate Fermentation (SSF)

ABSTRAK

Fermentasi substrat pepejal sisa nenas oleh Aspergillus flavus Link 44-1 telah diinisiatifkan di dalam penghasilan asid kojik. Asid kojik adalah asid organik yang digunakan secara meluas sebagai ramuan untuk produk dermatologi, penambah perisa dan juga sebagai ubat anti-keradangan. Dalam kajian ini, kesan kandungan kelembapan awal, pH dan masa inkubasi telah dikaji. Kandungan kelembapan awal untuk pengeluaran asid kojik melalui fermentasi substrat pepejal telah diperolehi pada 70% (v / w). Pengeluaran maksimum asid kojik juga dicapai pada pH 3.5 manakala julat optimum masa inkubasi bagi pengeluaran asid kojik yang tinggi pula dilihat antara 8 ke 16 hari. Kajian ini telah membuktikan potensi sisa nenas sebagai substrat alternatif untuk pengeluaran asid kojik oleh A. flavus Link 44-1.

Kata kunci: Asid kojik, Aspergillus flavus Link 44-1, Fermentasi substrat pepejal

1.0 INTRODUCTION

Pineapple is one of the important agricultural crops in Malaysia. Although the pineapple related industry is comparatively small as compared to other major export-based industries like rubber and palm oil, it also contributes to the economic growth of the country. Each year, pineapple canning industries throughout the region generate bulk amount of waste. Current disposal of pineapple waste poses tremendous environmental hazard due to the high content of organic material and suspended solid (Buckle, 1989).

Apart from their ecological pitfall, pineapple waste renders huge potentials to be exploited for various bioconversion processes. Nowadays, agro-industrial waste and by-product such as orange peel (Martin *et al.*, 2000), sugar cane bagasse (Silva *et al.*, 2002) and other food processing waste (Zheng *et al.*, 2000) are frequently used as alternative substrates for enzyme production or other products either via solid substrate fermentation (SSF) or submerged fermentation (SmF). This is due to the high amount of the residual compounds in such waste particularly starch, sugars, lignocellulose and others more. A number of bio-products can be produced depending on the microorganism used such as fungi and bacteria (Rosfarizan, 2000). In previous research works by Mustafa (2011), pineapple waste was used to cultivate *Aspergillus niger* via SSF and it was proven that pectinase was successfully produced from the cultivation. Other works reported in the literature on the use of pineapple waste as medium include the production of citric acid by *A. niger* (Kareem *et al.*, 2010; Kumar *et al.*, 2003).

One of the other bioproducts that can be initiated from pineapple waste via SSF is kojic acid. Kojic acid is an organic acid which is chemically known as 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one and 5-hydroxy-2-hydroxymethyl-4-pyrone (Kahn *et al.*, 1995). For years, this organic acid has high commercial value in various industrial fields such as medicine, agriculture and cosmetics. In cosmetics industry for example, the demand of kojic acid is increasing enormously with the growing presence of industries related to its applications. Kojic acid is highly produced by *Aspergillus* spp. and *Penicillium* spp. but *Aspergillus flavus* has always become the organism of choice due to its ability to produce high amount of kojic acid (Rosfarizan, 2000).

To date, there is no work yet reported on the use of pineapple waste as substrate for kojic acid production by *A. flavus* Link 44-1. Therefore, the emphasis of this research project was to study the production of kojic acid by *A. flavus* Link 44-1 using pineapple waste as substrate via SSF. The objectives of this study were:

- i. To study the usability of pineapple waste as substrate for kojic acid production by *A. flavus* Link 44-1 via SSF
- ii. To discover the optimum conditions for kojic acid production by *A. flavus* Link 44-1 via SSF

2.0 LITERATURE REVIEW

2.1 Pineapple waste

In Malaysia, pineapple industry is one of the oldest agro-based export-oriented industries. Johor is the major contributor to the pineapple industry in the country while in other states such as Selangor, Perak, Kelantan, Terengganu, Negeri Sembilan and Sarawak, pineapple are planted specifically for domestic fresh consumption.

The increasing production of pineapple yearly generates continuous accumulation of its waste which is either utilized as animal feed or disposed to the soil. This has led to serious pollution problems in the country due to the high amount of organic material and suspended solid which in turn cause high BOD level and severe pH condition. Despite the detrimental effects that the waste might pose, pineapple wastes have huge potentiality to be utilized in the production of variety of value added products. This is due to the notable amount of its beneficial residual compounds such as sugars and other nutrients.

The use of agro-industrial waste or by-product as substrate in bioconversion processes offers some advantages particularly in reducing the production cost which is often associated with expensive cost of raw materials. As well this effort can raise environmental concerns concerning the energy recycling and waste disposal among the citizens.

2.2 Solid substrate fermentation (SSF)

SSF is frequently applied as cultivation mean for agro waste-based bioprocess due to the close resemblance of the method to the natural condition of microbial cultivation. In recent years, SSF is widely applied for the production of various microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products.

SSF is defined as a process that involves the growth of microorganism on solids in the absence or near absence of free flowing water (Gabiatti *et al.*, 2006 and Pandey *et al.*, 2000). In this process, the substrate only possess little amount of moisture that can support growth and metabolism of microorganism.

Selection of proper substrate is one of the important aspects in SSF. Solid material acts as physical support and source of nutrients for the cultivation. The advantages of SSF over submerged fermentation (SmF) are higher end-concentration of products and stability, higher fermentation productivity, lower catabolic repression and lower demand on sterility due to low water activity used. Besides that, cultivation of microorganism specifically for insoluble substrate is also one of its advantages (Vidayalakshmi, 2009).

In previous works, submerged fermentation (SmF) is widely used for production of kojic acid (Rosfarizan *et al.*, 1998; Futamura *et al.*, 2001; Takamizawa *et*

al., 1996). However there is no work yet that has been reported on the use of SSF for kojic acid production from pineapple waste.

2.3 Kojic acid

Kojic acid is an organic acid that is naturally present in other substances such as the leftover residue from fermenting natural foods for example, rice, soy, wheat and pineapple (Sardjono, 1998). It is used largely as whitening agent and as UV light protector in cosmetics for many decades (Kobayoshi *et al.*, 1996). Kojic acid functions as an anti-melanosis since it inhibits polyphenol oxidase during post-harvest of agriculture products. Other than that kojic acid also serves as an important compound for other industries such as in medicine as anti-inflammatory and pain killer (Ozturk *et al.*, 2001), in agriculture as pesticides (Dowd, 2000) and also in food processing as flavor enhancer and anti-browning agent (Kaatz, 1999).

According to Rosfarizan *et al.*, (2000), kojic acid production can be divided into two phases which are growth phase and production phase. During the growth phase, amylolytic enzymes are secreted and kojic acid is synthesized during the secondary phase by the direct conversion from glucose throughout multistep reactions without any cleavage into small fragments. Kojic acid can be possibly produced from several kinds of carbon sources. Previous reports revealed the feasibility of kojic acid

production from corn starch (Futamura *et al.*, 2001), sago and potato starch (Rosfarizan *et al.*, 1998) and sucrose (Rosfarizan and Ariff, 2007).

2.4 *Aspergillus flavus* Link 44-1

Fungi that are responsible for the production of kojic acid are mainly *Penicillium* sp. and *Aspergillus* sp. of which *flavus-oryzae-tamaris* group are regarded as the best kojic acid producer (Amstein and Bently, 1953). Most of the filamentous fungi grow best in aerobic environment. Their growth depends on the temperature and water activity. Filamentous fungi are usually used in SSF compared to other microorganism due to their ability to sustain at low water activities (Molitoris, 2000).

The colonies of *A. flavus* are yellow to green or brown mould with a goldish to red-brown in colour. They are mostly saprophytic and grow excellently on the surface of carbon-rich substrate. Kojic acid productions by *A. flavus* have been reported by several researchers in the literature (Rosfarizan *et al.*, 1998; Ariff *et al.*, 1996).

Table 1: Production of kojic acid by *A. flavus* via SmF using different types of substrate

Substrate	Reference
Sodium acetate	Arnstein and Bentley (1956)
Arabinose	Arnstein and Bentley (1956) Basappa <i>et al.</i> (1970)
Glucose	Ariff <i>et al.</i> (1996), Kwak and Rhee (1991), Bajpai <i>et al.</i> (1982), Wan <i>et al.</i> (2005)
Sucrose	Rosfarizan and Ariff (2007)
Starch	Rosfarizan <i>et al.</i> (1998)

The prominent ability of several kojic acid producers such as *Aspergillus* sp. 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. In this study, *A. flavus* Link 44-1, which was locally isolated from morning glory flower, was used for the production of kojic acid from pineapple waste.

3.0 MATERIALS AND METHOD

3.1 Pre-treatment of substrate

Pineapple agricultural waste was collected from pineapple plantation site in Kota Samarahan, Sarawak. Firstly, pineapple waste was cleaned with running tap water and rinsed with distilled water. The pineapple waste was cut down into small pieces first before it was blended. After that, the blended product was dried in an oven for 72 hours (3 days) at 69°C or until a constant weight is achieved. Lastly, the dried substrate was milled and sieved before to be used in SSF.

3.2 Microorganism

The *Aspergillus flavus* Link 44-1 strain was obtained from Department of Bioprocess Technology, UPM. The strains of *A. flavus* Link 44-1 was subcultured on the Potato Dextrose Agar (PDA). The *A. flavus* Link 44-1 was grown for 7 days at 30°C. Inoculum was prepared in the form of spore suspension with standardized concentration of 1×10^5 spore/ml (Rosfarizan, 2000).



Figure 1: 7-day old *A. flavus* Link 44-1 on PDA

3.3 Solid Substrate Fermentation (SSF)

5 g pineapple waste was used as substrate in each culture. The culture was inoculated with spore suspension of 1×10^5 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 the SSF of pineapple waste by *A. flavus* Link 44-1 were investigated. The details are as follow.

3.3.1 Effect of initial moisture content on SSF of pineapple waste by *A. flavus* Link 44-1

The substrate for the cultivation was adjusted to approximately of 60%, 65%, 70%, 75% and 80% (v/w) with sterile distilled water prior to incubation.

3.3.2 Effect of pH on SSF of pineapple waste by *A. flavus* Link 44-1

The effects of pH on kojic acid production from pineapple waste by *A. flavus* Link 44-1 via SSF were determined by testing different levels of initial pH on the cultivation. The initial pH was adjusted to pH; 2.5, 3.0, 3.5, 4.0 and 4.5.

3.3.2 Effect of time of incubation on SSF of pineapple waste by *A. flavus* Link 44-1

The effect of incubation time on kojic acid production from pineapple waste was investigated by setting the cultivation at different period of time. The cultures were collected 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).

3.4 Extraction

Firstly, 50 ml of distilled water was added to the sampled culture. The slurry suspension was centrifuged at 6000 rpm for 20 minutes at 4°C (Conti *et al.*, 2001). After that, the suspension was filtered through 0.45 µm filter prior to reducing sugar and kojic acid assays. The assays were prepared in duplicates and the results for the assays were expressed as means of duplicate values.

3.5 Reducing sugar analysis

In this work, the Dinitrosalicylic acid (DNS) method (Miller, 1959) was employed 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. DNS reagent was prepared by dissolving 1g 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 was added with 1 ml of Roschell salt and the absorbance was read at 575 nm by using spectrophotometer (UV mini-1 240v, Shibasu Corporation, Japan).

3.6 Kojic acid analysis

Kojic acid was quantified by using colorimetry method (Bentley, 1957) 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 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 mL of 0.1N 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, Shibasu Corporation, Japan) at a wavelength of 500 nm. The kojic acid equivalent was determined from the kojic acid standard curve as shown in Appendix. The colorimetry method was widely used in the kojic acid analysis due to its flexibility compared to other methods.

3.7 Statistical Analysis

All the experimental data were statistically analyzed by using Analyse-it Statistical Software (version 2.26 Analyse-it Inc, Leeds, UK). The Tukey method was used for comparing the mean values via One-Way Analysis of Variance (ANOVA).

4.0 RESULTS AND DISCUSSIONS

4.1 Effect of initial moisture content on SSF of pineapple waste by *A. flavus* Link 44-1

In examining the effect of initial moisture content on SSF of pineapple waste by *A. flavus* Link 44-1, five different levels were tested which were 60%, 65%, 70%, 75% and 80% (v/w). **Figure 2** and **Figure 3** show the profiles of reducing sugar and kojic acid over time respectively at different concentrations of initial moisture content. Drastic reduction of reducing sugar was seen after 8 days (192 hours) of cultivation. This corresponded to the notable increase of kojic acid production which also occurred on 8th day but then decreased after 14th day (336 hours) and onwards.

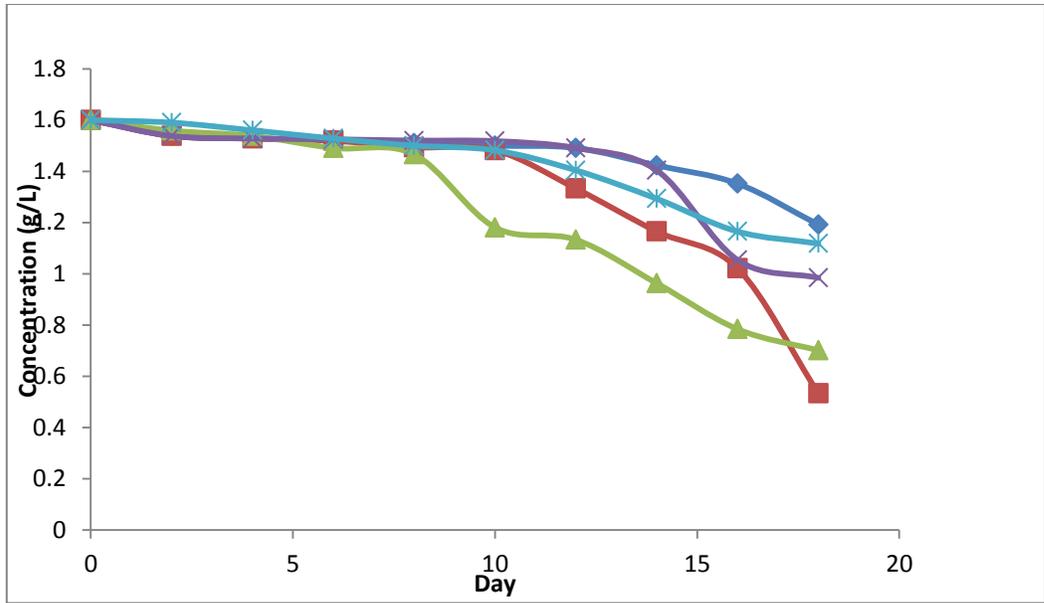


Figure 2: Effect of initial moisture content on reducing sugar consumption by *A.flavus* Link 44-1 via SSF of pineapple waste. (◆) 60% (v/w); (■) 65% (v/w); (▲) 70% (v/w); (x) 75% (v/w) and (⊗) 80% (v/w).

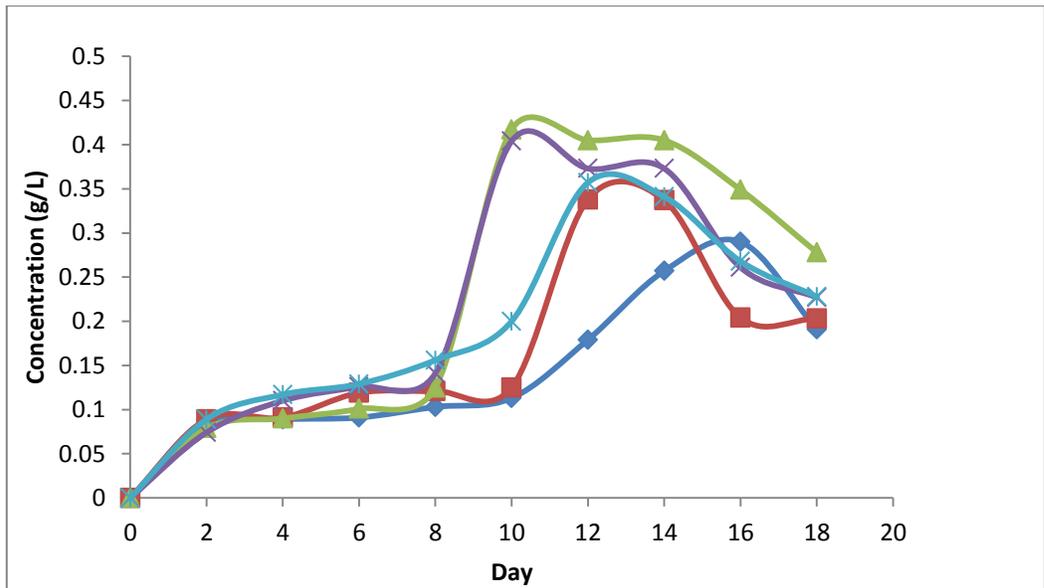


Figure 3: Effect of initial moisture content on kojic acid production by *A.flavus* Link 44-1 via SSF of pineapple waste. (◆) 60% (v/w); (■) 65% (v/w); (▲) 70% (v/w); (x) 75% (v/w) and (⊗) 80% (v/w).

Throughout the range of initial moisture content tested from 60% to 80% (v/w), the highest production kojic acid was generally found at 70% (v/w) with the maximum concentration of 0.4145 g/L. This was found to be in line with its highest rate of reducing sugar consumption as compared to other levels of initial moisture content as illustrated in **Table 2**.

Table 2: Comparison of maximum production of kojic acid from pineapple waste by *A.flavus* Link 44-1 via SSF at different initial moisture content levels

Initial moisture content (v/w)	Maximum production of kojic acid (g/L)
60%	0.2895 ^a
65%	0.3338 ^b
70%	0.4145 ^c
75%	0.3730 ^d
80%	0.3565 ^{b,d,e}

Similar superscript letter represents no significant variation of the mean values ($p < 0.05$)

Comparison of the maximum production of kojic acid from pineapple waste by *A. flavus* Link 44-1 via SSF at each level of initial moisture content is outlined in **Table 2**. The result shows that there was a significant rise of maximum kojic acid production when the concentration of initial moisture content was increased from 60% to 75% (v/w). On the other hand, further increase of initial moisture content from 75% to 80% (v/w) exhibits no significant change of the maximum kojic acid production. From these results, it can be suggested that, further enhancement of kojic acid production by *A. flavus* Link 44-1 via SSF of pineapple waste can be attained by optimizing the