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

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Bachelor of Science with Honours
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Production of Kojic Acid via Solid State Fermentation (SSF) of Pineapple Waste by
Aspergillus flavus NSH9

Nadia Dayana Sikem

A progress submitted in partial fulfilment of the degree of Bachelor of Science with
Honours
(Resource Biotechnology)

Supervisor: Miss Nurashikin binti Suhaili
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Resource Biotechnology
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2013
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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.
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
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<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalicylic acid</td>
</tr>
<tr>
<td>SSF</td>
<td>Solid state fermentation</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>°C</td>
<td>Degree of celsius</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>g/L</td>
<td>Gram per liter</td>
</tr>
<tr>
<td>v/w</td>
<td>Volume per weight</td>
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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
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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


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 Kareem 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 delbrueckii* (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 (Bankoff 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>
<thead>
<tr>
<th>Product</th>
<th>Microorganism</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioprotein</td>
<td>Phanerochaete chrysosporium</td>
<td>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 biocconversion using lower concentration (%w/v) of media components (PSS- 1.0, KH₂PO₄- 0.10, and NH₄H₂PO₄- 0.25)</td>
<td>Jamal et al.,</td>
</tr>
<tr>
<td>(function as protein</td>
<td>PC-13 (basidomytes)</td>
<td></td>
<td>(2009)</td>
</tr>
<tr>
<td>supplementation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for human and animal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>Aspergillus niger</td>
<td>Maximum citric acid of 60.0 g/kg of pineapple waste was obtained under optimum conditions.</td>
<td>Kareem et al.,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2010)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Zymomonas mobilis</td>
<td>The ethanol yield from pineapple waste was 92% of the theoretical yield and the productivity was 2.81 g·l⁻¹·h⁻¹.</td>
<td>Tanaka et al.,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1999)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Lactobacillus delbrueckii</td>
<td>The highest yield was 85.65 % achieved at 40°C, pH of 6.00, and 52.5 g/l sugar concentration with 5 g/l yeast extract.</td>
<td>Abdullah</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2010)</td>
</tr>
<tr>
<td>Phenolic antioxidants</td>
<td>Rhizopus oligosporus</td>
<td>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.</td>
<td>Masimir</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2011)</td>
</tr>
</tbody>
</table>
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 environmentally-friendly (Solangé 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 (Vidyalakshmi, 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>
<thead>
<tr>
<th>Substrate</th>
<th>Microorganism</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatinized and hydrolyzed sago starch</td>
<td><em>Aspergillus flavus</em></td>
<td>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 hydrolysate (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.</td>
<td>Rosfirtizan et al. (1998)</td>
</tr>
<tr>
<td>Partially hydrolyzed raw corn starch</td>
<td><em>Aspergillus oryzae</em> MK-107-39</td>
<td>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.</td>
<td>Futamura et al. (2001)</td>
</tr>
<tr>
<td>Glucose and Polypepton</td>
<td><em>Aspergillus oryzae</em></td>
<td>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/a value of 164.7 h⁻¹.</td>
<td>Takamizawa et al. (1996)</td>
</tr>
<tr>
<td>Sucrose</td>
<td><em>Aspergillus candidus</em></td>
<td>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.</td>
<td>Wei et al. (1991)</td>
</tr>
<tr>
<td>Glucose</td>
<td><em>Aspergillus parasiticus</em></td>
<td>The highest level of kojic acid (34.38 g L⁻¹) was obtained by <em>A. parasiticus</em> 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).</td>
<td>El-Aasar (2006)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic morphology</td>
<td>Length usually less than 1 mm, heavy walled, uncolored, and coarsely roughened</td>
</tr>
<tr>
<td>Macroscopic morphology of vesicles</td>
<td>Elongated when young, later becoming sub-globose or globose (round, radiate head), varying from 10 to 65 μm in diameter.</td>
</tr>
<tr>
<td>Macroscopic morphology of phialides</td>
<td>Biseriate (vesicles produces sterile cells known as metulae that support the conidiogenous phialides) or uniseriate. The primary branches are up to 10 μm, and the secondary branches up to 5 μm.</td>
</tr>
</tbody>
</table>

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 Sml*. 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 262 g/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 subsirate 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.](image)
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 \textit{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 \textit{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 \textit{A. flavus} NSH9

The effect of pH on kojic acid production from pineapple waste by \textit{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 \textit{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), 114 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 \textit{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 (FeCl₃) solution. FeCl₃ was prepared by dissolving 1 g of FeCl₃.6H₂O 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 Hivar prepacked column RT 250-4 Lichrosorb RP-18 (10µm).