EVALUATION OF SAGO 'HAMPAS' AS POTENTIAL CARBON SOURCE FOR KOJIC ACID PRODUCTION BY *ASPERGILLUS FLAVUS* LINK 44-1

Fatin Nadiah Binti Aziz (23485)

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Supervisor: Miss Nurashikin Bt Suhaili

Co-supervisor: Puan Dayang Salwani Bt Awang Adeni

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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LIST OF ABBREVIATIONS

AGU/ml	Antigen Unit per Milliliter
DNS	Dinitrosalycylic acid
g/L	gram over Litre
MEA	Malt Extract Agar
mg	milligram
mL	millilitre
nm	nanometer
pH	A measurement of the acidity or alkalinity of solution [p stands for "potenz" which means the potential to be while H stands for Hydrogen]
rev/min	revolution per minutes
UV	Ultra Violet
v/v	volume over volume

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Evaluation of Sago 'Hampas' As Potential Carbon Source for Kojic Acid Production by *Aspergillus flavus* Link 44-1

Fatin Nadiah Binti Aziz

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Sago 'hampas' is one of the abundantly available lignocellulosic wastes that renders huge potentiality to be applied as alternative substrate in the production of various bioproducts. In this present work, the use of sago 'hampas' hydrolysate as feedstock for production of kojic acid by *Aspergillus flavus* Link 44-1 was tested and glucose was referred as a control. Hydrolysis using 0.3% (w/v) Dextrozyme produced a maximum yield of 0.59 g reducing sugar/ g sago 'hampas' which was then applied as substrate for kojic acid production. The kojic acid fermentation was carried out in batch culture with 100 mL working volume at pH 3, 140 rev/min and 30°C. The effects of four different concentrations of inoculums namely 10^4,10^5,10^6 and 10^7spores/ml on the fermentation were examined. The use of sago 'hampas' hydrolysate exhibited the highest kojic acid production up to 5.88 g/L with productivity of 0.0355 g/L.h when the spore concentration of 10^7spores/ml was applied as inoculum. This result indicates the feasibility of sago 'hampas' as potential carbon source for the production of kojic acid by *A. flavus* Link 44-1.

Keywords: *Aspergillus flavus* Link 44-1, kojic acid, sago 'hampas', hydrolysate

ABSTRAK

Sagu 'hampas' merupakan salah satu sisa lignoselulosik yang didapati dengan banyaknya, menyebabkan ia sangat berpotensi untuk diaplikasikan sebagai substrat alternatif dalam penghasilan pelbagai bioproduk. Dalam hasil kerja ini, pengunaan sagu 'hampas' hidrolisat sebagai bahan mentah bagi penghasilan asid kojik oleh *Aspergillus flavus* Link 44-1 telah diuji dan glukosa telah digunakan sebagai kawalan. Hidrolisis dengan menggunakan 0.3% (b/i) Dextrozim telah memberi peghasilan maksimum sebanyak 0.59g gula mengurang/g sagu 'hampas' yang kemudiannya diaplikasikan sebagai substrat untuk penghasilan asid kojik. Fermentasi asid kojik telah dijalankan dalam kultur kumpulan dengan isipadu 100mL pada pH 3, 140 rev/min dan 30°C. Kesan-kesan keatas empat kepekatan inukulum yang berbeza iaitu 10^4,10^5,10^6 dan 10^7spores/ml dalam fermantasi telah dikaji. Pengunaan sagu 'hampas' hidrolisat telah menunjukkan penghasilan asid kojik yang tertinggi sehingga mencapai 5.88 g/L dengan produktiviti sebanyak 0.0355 g/L.j bila 10^7spores/ml telah digunakkan sebagai inukulum. Keputusan ini menandakan sagu 'hampas' berkeupayaan untuk dijadikan sebagai sumber karbon yang berpotensi bagi penghasilan asid kojik oleh *A flavus* Link 44-1.

Kata kunci: Aspergillus flavus Link 44-1, asid kojik, sagu 'hampas', hidrolisat

1.0 INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) serves as an important metabolite for many industrial purposes. Such as in medicine as pain killer, and anti-inflammatory drug (Kayahara *et al.*, 1990), antibacterial (Kotani *et al.*, 1976), in food production as antioxidant and flavour enhancer (Chen *et al.*, 1991), in cosmetic products as whitening agent, UV protector (Ohyama and Mishima, 1990; Noh *et al.*, 2009) and radioprotective agent (Emami *et al.*, 2007). Kojic acid is produced mainly by *Aspergillus* spp. of which among all, *flavus-oryzae-tamarii* groups were revealed as the best kojic acid producer (Gould, 1938).

Nowadays, enormous industrial benefits from kojic acid have increased its demand and commercial values for large scale production. However its production is often limited due to the high cost of raw materials which necessitates the need to switch to other economical yet potential alternative sources. The increasingly opted way to cut down the production cost is by using potential agro waste and one of promising substrate identified is sago 'hampas'. This is due to its composition that is made up of approximately 66% starch and 14% fibre on a dry weight (Chew and Shim, 1993). Hence, sago 'hampas' presents huge potentially to be utilized as feedstock for bioprocess and this is further supported by its wide availability of sago in Sarawak.

Presently, no work has been reported yet on the use of sago 'hampas' for kojic acid production and thus there is a need to test the feasibility of sago 'hampas' as one of the potential, economical and environmental friendly carbon sources for kojic acid production. The use of sago hampas as a raw material or carbon source for fermentation is seen as economically promising as its use will cut down the production cost of kojic acid.

Aspergillus flavus Link 44-1 that was isolated from morning glory flower has been proven capable to use starch as carbon source for kojic acid production (Rosfarizan *et al.*, 1998). In this study, the effectiveness of the sago 'hampas' as carbon source for kojic acid production by *A.flavus* Link 44-1 was investigated. Therefore the objectives of this study were:

1) To evaluate the effectiveness of sago 'hampas' hydrolysate as carbon source for kojic acid production in comparison with other carbon sources.
2) To investigate the effect of inoculums size on kojic acid production by *A.flavus* Link 44-1using sago hampas hydrolysate as substrate.

2.0 LITERATURE REVIEW

2.1 Sago 'Hampas'

Sarawak is the biggest producer of sago (*Metroxylon sagu*) in Malaysia (Awg-Adeni *et al.*, 2010), where it is estimated that 44,700 tonnes of sago starch are exported to Peninsular Malaysia, Asian and other countries yearly (Sarawak agriculture statistic, 2007). In comparison with other starch-producing crops such as rice, corn, wheat and potato, sago yields the highest amount of starch with the annual production of approximately 25 tonnes (Ishizaki, 1997). The by-product generated from sago starch processing industry includes bark of sago trunk and also fibrous pith residues known as 'hampas' as well as liquid residue which is the waste water (Awg-Adeni, 2010). Unfortunately, the improper disposal of these residues often leads to many environmental problems as the residues are normally washed off into nearby streams. Studies have shown that, these lignocellulosic by-products can serve as valuable resources for various bioconversion processes. Besides, this initiative is seen as one of the ways to promote the conservation of the environment.

In this study our main concern was to use sago 'hampas' as substrate for kojic acid production by *A.flavus* Link 44-1. As reported in the literature, sago hampas contains about 66% starch on a dry weight basis of which approximately 25% is made up of lignin (Chew and Shim, 1993). According to Awg-Adeni *et al.* (2010), by means of acid or enzymatic hydrolysis, the high amount of starch in sago hampas can be converted to fermentable sugar which can be further used as substrate for fermentation process.

2.2 Kojic Acid

Kojic acid is known by several chemical names namely 5-Hydroxy-2 (hydroxymethyl)-4H-pyrane-4-one, 4H-Pyran-4-one, 5-hydroxy-2-(hydroxymethyl-5-Hydroxy-2 hydroxymethyl -4-pyrone, and 2-hydroxymethyl-5-hydroxy-4-pyrone. This compound is white to light yellow crystalline in the form of powder with molecular weight of 142.12 g/L. It contains impurities that may include heavy metals (10 mg/kg max., not specified) and arsenic (4 mg/kg max.)(IARC Monograph, 2001). Moreover this compound is soluble in water (43.85 g/l); acetone, ethyl acetate and pyridine (IARC Monograph, 2001), while slightly soluble in ethanol and insoluble in diethyl ether, chloroform or benzene (Lide and Milne, 1996; Budavari, 2000). The melting point of kojic acid is 153.5°C (Lide and Milne, 1996). In addition, kojic acid is a multifunctional compound that is capable to react at every position on its ring. There are several industrial chemicals that are produced from kojic acid such as metal chelates, ethers, azodyes and pyridines (Ichimoto *et al,* 1965; Wilson, 1971).

As stated by SCCP (2008), kojic acid is a fungal metabolite which is produced by many species of *Aspergillus*, *Penicillium,* and *Acetobacter*. In cosmetics industry, spectrophotometric and chromatographic methods demonstrated that kojic acid is capable to reduce *o*-quinones to diphenols that able to prevent the formation of final pigment (melanin). Due to its reactivity that can be slowed down as well as reversible competitive inhibition of tyrosinase, kojic acid is preferably applied as skin-lightening agent in dermatological products with dosage of 2-4%.

Other than being used in cosmetic field, kojic acid has also been utilized in many industrial applications such as in pharmaceutical field for production of anti-neoplastic compound (Novotny *et al.,* 1999), flavour enhancer (Wood, 1998), antibiotic (Kotani *et al.*, 1976) and antifungal activities (Kayahara *et al.,* 1990; Balaz *et al.,* 1993). There are

many works that have been reported on the production of kojic acid from *Aspergillus* spp, and one of the most potential producers is *Aspergillus flavus* since it is capable to produce kojic acid in large quantity (Bajpai *et al.*, 1981).

2.3 *Aspergillus flavus* spp.

Aspergillus species are group of molds which are easily found in a broad range of environment (Bennett, n.d). The fungi are classified as saprophytic which develop on dead or decaying organic matter. More than 100 species of *Aspergillus* have been identified whereby several of them are *A. niger, A. oryzae, A. versicolor and A. flavus.* Most of filamentous fungi grow aerobically on organic matter. Among the factors that influence the growth of *Aspergillus* species are pH (Esteban *et al.*, 2006), water activity and temperature (Romero *et al.*, 2007). Table 1 shows some works as reported in the literature on several kinds of *Aspergillus* sp. that are capable to produce kojic acid.

Table 1: Previous works on kojic acid production using various types of *Aspergillus* spp.

Microorganism	Previous Research
A. parasiticus	El-Aasar (2006) Gad (2003) Nandan and Polasa (1985) Coupland and Niehaus (1987)
A. tamarii	Gould (1938)
A. oryzae	Futamura *et al.* (2001) Lin *et al.*, (2001) Kitada *et* al. (1967) Kwak and Rhee (1992)
A. flavus	Ariff *et al.* (1997) Rosfarizan *et al.* (1998), Rosfarizan *et al.*, (2000); Rosfarizan *et al.* (2002); Rosfarizan *et al.* (2006) Basappa *et al.* (1970)

A.flavus grows widely in the soil and also on dead plant and animal tissue (Chang & Ehrlich, 2010). The growth of the colonies is optimized at 37°C. The colonies usually present in yellow to dark yellowish-green, with a diameter of 6 to 7 cm in 10 to 14 days (Ruiqian *et al.*, n.d). As the fungi enter late stationary phase the colonies present in dark green colour (Ruiqian *et al.*, n.d). Figure 2 shows conidia of *A.flavus* mold, with budding conidiospores and the releasing conidia. Figure 3 on the other hand illustrates a picture of *A. flavus* spores from scanning electron microscope.

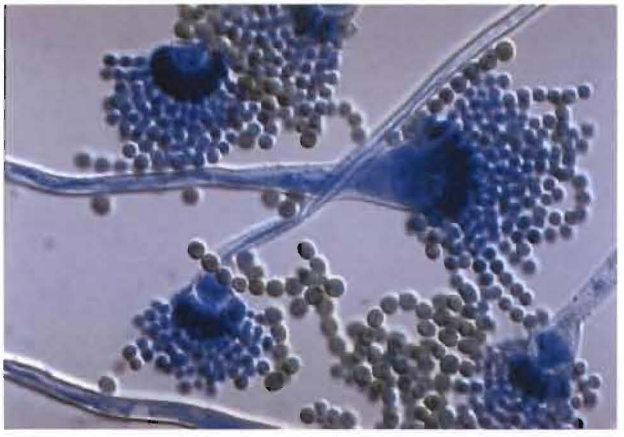

Figure 1: Microscopical view conidia of *A. flavus* mold. Retrieved on 17 October, 2011 from http://www.xn--krinfo-wxa.hu

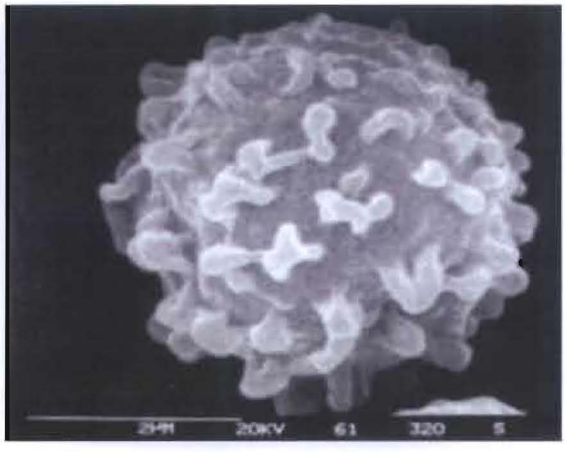

Figure 2: *A. flavus* spores viewed from Scanning Electron Microscope (Rodrigues *et al.*, 2007).

Suitable medium formulation and appropriate maintenance of culture condition can inhibit the production of aflatoxins (Madihah, 1996). Furthermore as revealed by to Basappa *et al.* (1970), kojic acid is not an intermediate in the synthesis of aflatoxins; since kojic acid and aflatoxins follow different pathways.

3.0 MATERIALS AND METHOD

3.1 Microorganism

The *A. flavus* Link 44-1 strain used in this work which was isolated from morning glory flower was obtained from Universiti Putra Malaysia. The strain was subcultured on Malt Extract Agar (MEA) and incubated at 30°C. The *A. flavus* Link 44-1 spores were then harvested from the media by pouring sterile 0.1% Tween-80 on surface of media to wash off the spores. The spore concentration is measured by counting with a hemacytometer under a microscope. The spore count was conducted once the substrate is ready. A standard inoculums of 10% (v/v) was used in entire experiment.

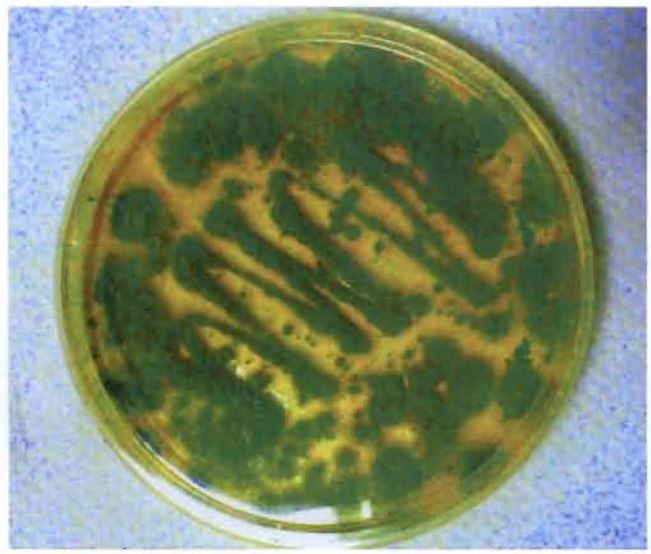

Figure 3: 7 day-old *A. flavus* Link 44-1

3.2 Pretreatment of Substrate

The sago 'hampas' used in this work was obtained from Hardsen Sago Mill, Pusa, Sarawak. Prior to fermentation, the substrate was first treated via 2 steps namely physical treatment and enzymatic hydrolysis.

3.2.1 Physical Treatment

Series of steps of physical treatment were carried out. Firstly, the sago effluent sample was filtered. The filtered obtained was then blended and sieved. The resulting sago 'hampas' was then dried in an oven at 60°C until a constant weight is achieved.

Figure 4: Pre-treated sago hampas.

3.2.2 Enzymatic Hydrolysis from Starch Constituent of Sago 'hampas'

Sago hampas was mixed with KH_2PO_4 buffer pH 4 with the ratio of 6 g sago hampas to 100ml of buffer. The mixture was stirred for 10 minutes for gelatinization to occur. Prior to that, the buffer was heated until 80°C. The mixture

was left to cool until 60°C before enzyme 30μl Dextrozyme (per 10 gram of sago hampas) was added. The enzyme Dextrozyme was added for saccharification purpose. The enzyme supplied by Novo Nordisk; enzyme Dextrozyme (mixture of glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*, 225 AGU/ml). The reaction was incubated with manual stirring for 2 hours at 60°C. Then the mixture was allowed to cool to room temperature before it was filtered prior to be applied as substrate. The residual fibre was dried in an oven for 24 hours for determination of starch conversion.

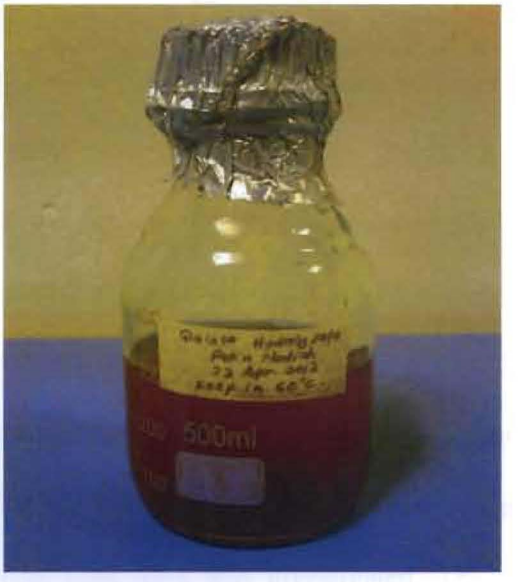

Figure 5: Sago 'hampas' hydrolysate

3.2.3 Quantification of Reducing Sugar Content

The resulting sugar hydrolysate from enzymatic hydrolysis of sago 'hampas' was quantified using Dinitrosalycylic acid (DNS) method (Miller, 1959). 3 mL of DNS reagent added to 3mL of the sugar hydrolysate sample and the reaction mixture was heated at 90°C for 15 minutes. Next, 1 mL of a 40% (w/v) potassium sodium

tartrate (Rochelle salt) solution was added to stabilize the colour of the mixture. Finally, the mixtures were allowed to settle at room temperature by cooling them in a cold water bath. The absorbance of the reaction mixture was read at 575 nm using spectrophotometer (model UV Mini-1240, Shimazu UV-Vis Spectrophotometer, Japan). The concentration of the sugar hydrolysate was determined by using glucose as a standard curve (Appendix A).

3.3 Fermentation

Fermentation process was carried out in 250 ml shake flask with working volume of 100 ml. Other than the carbon source, the optimized media as described by Madihah *et al.* (1993), that contain $5gL^{-1}$ yeast extract, $1gL^{-1}$ KH_2PO_4, $0.5gL^{-1}$ $MgSO_4.7H_2O$ and $10gL^{-1}$ methanol was used. In this work, sago 'hampas' was tested for its feasibility as carbon source for kojic acid production and glucose was used as a control. The cultivation was carried out at optimized cultural conditions whereby the pH of the culture was initially adjusted to 3.0 while the temperature and agitation rate were maintained at 30 °C and 140 rev/min respectively (Ariff *et al.*, 1996). The effect of different sizes of inoculum on kojic acid fermentation by *A.flavus* Link 44-1 was investigated. The four different levels of inoculums size tested were 10^4,10^5,10^6 and 10^7 spores/ml and were counted using haemocytometer. All experimental runs were done in triplicate.

3.4 Analytical Methods

During the fermentation, 5 mL of samples was drawn out aseptically in every 48 hours. The analyses performed were cell dry weight determination, reducing sugar analysis and kojic acid analysis.

3.4.1 Cell Dry Weight

The pre weighed microfiber filter paper was used to filter the sample. The residue was dried at 60 °C in an oven for 2 days or until a constant weight is attained. The difference in weight of the filter paper before filtration and after a constant weight of residue is reached is equivalent to the weight of dry cell. The biomass concentration was determined by dividing the dry cell weight with the volume of culture suspension to be filtered. The resulting supernatant on the other hand was used for reducing sugar and kojic acid determination (Rosfarizan *et al.*, 1998).

3.4.2 Reducing Sugar Analysis

Reducing sugar of the samples was determined by DNS method (Miller, 1959) as described in section 3.2.3.

3.5.3 Kojic Acid Analysis

Kojic acid was analysed by using colorimetry method as described by Bently (1957). The assay is based on the reaction between kojic acid and $FeCl_3$ where 1 mL of diluted sample was mixed with 1 mL of ferric chloride, ($FeCl_3$) solution. The $FeCl_3$ solution was prepared by dissolving 1 g of $FeCl_3.6H_20$ in 100 mL of 0.1N HCl (Rosfarizan *et al.*, 2000). The reaction between the functional group of hydroxyl and phenolic in the samples results in reddish purple mixture and the

absorbance of the reaction mixture was measured using spectrophotometer (model UV Mini-1240, Shimazu UV-Vis Spectrophotometer, Japan) at a wavelength of 500 nm. The concentration of kojic acid in the sample was quantified based on the standard curve by using pure kojic acid (Appendix B).The colorimetry method widely employed in the history of kojic acid research (Takamizawa *et al.*, 1996; Ogawa *et al.*, 1995; Bajpai *et al.*, 1982) due to its flexibility and simplicity as compared to other methods.

3.5 Statistical Analysis

All the experimental data were statistically analyzed by using Analyse-it Statistical Software (version 2.26 Analyse-it, Inc., Leeds, UK). The mean values were compared by applying Tukey's Method via One way Analysis of Variance (ANOVA).

4.0 RESULTS AND DISCUSSION

4.1 Production of sago 'hampas' hydrolysate via enzymatic hydrolysis

Prior to kojic acid fermentation, enzymatic hydrolysis of sago 'hampas' was carried out in order to produce its hydrolysate which was further used as feedstock in the cultivation of *A.flavus* Link 44-1. A notable amount of sugar was successfully attained through the recycling method of enzymatic hydrolysis. The conversion workout for the yield of reducing sugar from sago 'hampas' is shown in Appendix C.

In this work, a yield of 0.59 g reducing sugar/ 1 g sago hampas was successfully achieved. The initial concentration of sago 'hampas' hydrolysate used in all experimental runs in the present work was 33.2 g/L. Rosfarizan *et al.* (1998), in their previous study proved the usability of sago starch as substrate for kojic acid production and a maximum production of 5.12 g/L was successfully attained via submerged fermentation. To date, the use of sago 'hampas' hydrolysate has not yet been reported for the production of kojic acid.

4.2 Performance of kojic acid fermentation by *A.flavus* Link 44-1 using sago 'hampas' as substrate.

The effect of using sago 'hampas' as substrate on kojic acid fermentation by *A.flavus* Link 44-1 was evaluated in terms of the biomass production, substrate consumption and kojic acid production. Glucose which was revealed as the most suitable type of carbon source for kojic acid production (Rosfarizan *et al.,* 1998) was used as control in this work. The

cultivation was carried out at four different sizes of inoculums ranging from 1×10^4 spores/ml to 1×10^7 spores/ml.

4.2.1 Biomass production

In both types of cultures that used sago 'hampas' hydrolysate and glucose as substrate, cells grew in the form of spherical pellet. However, pellets in culture that employed sago 'hampas' hydrolysate was comparatively bigger in their diameter as compared to culture that used glucose. Different types of substrate adopted for cultivation might influence the morphological characteristics of the culture.

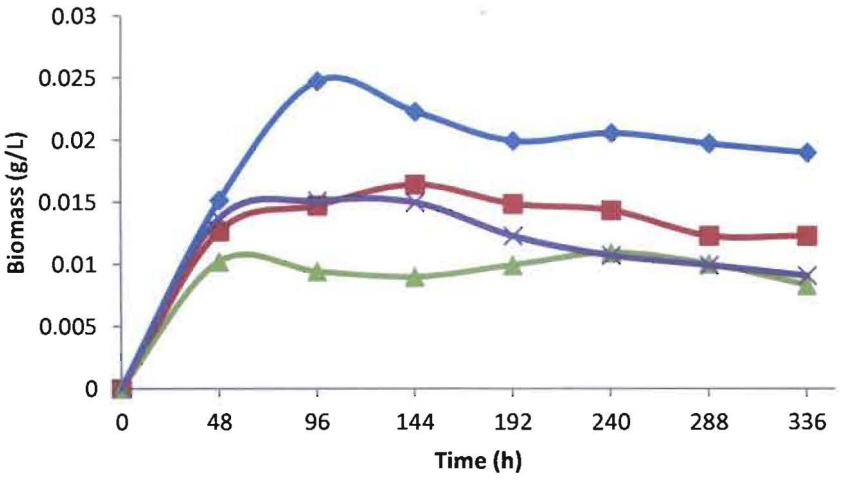

Figure 6: Time course of biomass concentration during kojic acid fermentation by *A.flavus* Link 44-1 using sago hampas hydrolysate. (x) 1×10^4 spores / ml; (▲) 1 x 10^5 spores / ml;(■) 1 x 10^6; spores / ml; (◆) 1 x 10^7 spores / ml

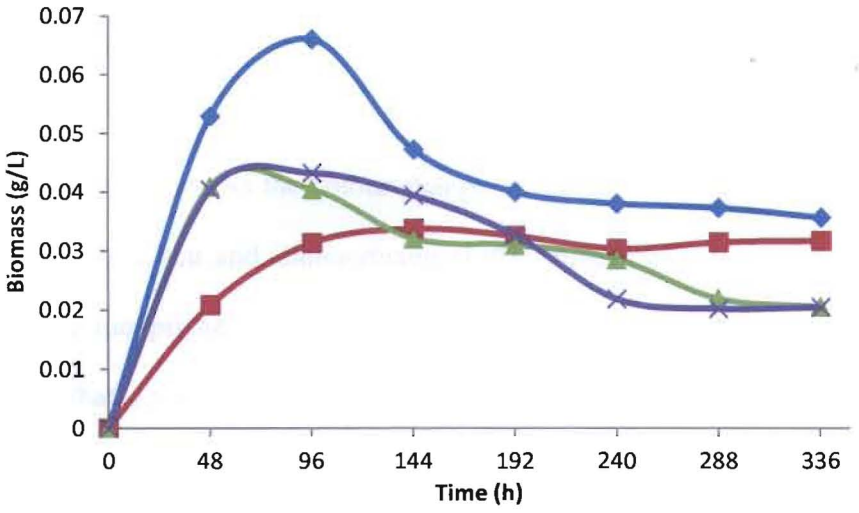

Figure 7: Time course of biomass concentration during kojic acid fermentation by *A.flavus* Link 44-1 using glucose. (x) 1×10^4 spores / ml; (▲) 1 x 10^5 spores / ml ;(■) 1 x 10^6 spores / ml; (◆) 1 x 10^7 spores / ml

Figure 6 and Figure 7 show the time profiles of biomass concentration during *A.flavus* Link 44-1 cultivation using sago 'hampas' and glucose respectively which were tested at four different sizes of inoculums. In general, the cell growth profile exhibited by cultivation that employed sago 'hampas' as substrate did not vary considerably with glucose-based culture. Stationary phase was reached approximately after 144 hours of incubation for most of the inoculums size studied. The maximum biomass concentration obtained in kojic acid fermentation using sago 'hampas' and glucose were 0.025 g/L and 0.066 g/L respectively. Both peak values were exhibited when the inoculums concentration of 1×10^7 spores was applied. The higher biomass concentration attained in kojic acid fermentation using glucose as substrate was expected since glucose was revealed as the most favourable carbon source for *A.flavus* growth (Rosfarizan *et al.*, 2000). In contrast, sago 'hampas' hydrolysate contains other types of sugar besides glucose and not all of sugars present might favourably support the growth of *A.flavus* Link 44-1.