BIODEGRADATION STUDIES OF SAGO HAMPAS BY ASPERGILLUS SPP.

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This thesis is submitted in partial fulfillment of the requirement for the Degree of Bachelor of Science with Honours (Resource Biotechnology)

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Declaration

I hereby declare that this thesis entitled “Biodegradation Studies of Sago Hampas by Aspergillus spp.” is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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<td>Bovine serum albumin</td>
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<td>DNS</td>
<td>3,5-dinitrosalicyclic acid</td>
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<tr>
<td>g</td>
<td>gram</td>
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<td>g/L</td>
<td>Gram per litre</td>
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<td>μ</td>
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<td>Nm</td>
<td>Nanometer</td>
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<td>OD</td>
<td>Optical Density</td>
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<td>PDA</td>
<td>'Potato Dextrose Agar</td>
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<td>rpm</td>
<td>rotation per minutes</td>
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<td>SSSF</td>
<td>Solid State Fermentation</td>
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<td>Spp.</td>
<td>Species</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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ABSTRACT

Sago hampas is released from sago processing factory after starch extraction. These wastes have been disposed of and leading to environmental problems. Therefore, this project aims to study the biodegradation of sago hampas by *Aspergillus* species. *Aspergillus* species plays a significant role in producing extracellular enzymes. Two strains of fungi, *Aspergillus niger* and *Aspergillus flavus* were used in the solid state fermentation where sago hampas is used as substrate. Three analytical analysis were done to determine the rate of biodegradation, including enzyme assays, protein determination and phenol-sulfuric acid assay. Based on the data obtained, the fermentation with *A. flavus* shows the highest rate of degradation which is 42% as compared to *A. niger* (40%) and *Aspergillus* consortia (33.6%). It also shows the highest enzymes activity of cellulase (0.053 U/ml) and amylase (0.082 U/ml) produced by *A. flavus*. The results from this work present the promising capability of *A. flavus* to biodegrade sago hampas.

Keywords: Biodegradation, *Aspergillus niger*, *Aspergillus flavus*, Solid-state fermentation

Sagu hampas dikeluarkan dari kilang pemprosesan sagu selepas pengekstrakan kanji. Sisa yang telah dilupuskan membawa kepada masalah alam sekitar. Oleh itu, projek ini bertujuan untuk mengkaji tahap biodegradasi sagu hampas oleh *Aspergillus* spesies. *Aspergillus* spesies memainkan peranan penting dalam menghasilkan enzim-enzim. Dua jenis kulat, *Aspergillus niger* dan *Aspergillus flavus* telah digunakan dalam fermentasi keadaan pepejal di mana hampas sagu digunakan sebagai substrat. Tiga analisis analitikal telah dilakukan untuk menentukan tahap biodegradasi iaitu termasuklah asat enzim, penentuan protein dan asaifenol asid. Berdasarkan kepada data-data yang diperolehi, fermentasi dengan *A. flavus* menunjukkan kadar degradasi tertinggi iaitu 42% jika dibandingkan dengan *A. niger* (40%) dan konsortium *Aspergillus* (33.6%). Data juga menunjukkan tahap aktiviti enzim yang tinggi oleh *A. flavus* iaitu aktiviti selulase (0.053 U/ml) dan aktiviti amilase (0.082 U/ml). Keputusan projek ini menunjukkan keupayaan *A. flavus* untuk proses biodegradasi sagu hampas.

Kata kunci: Biodegradasi, *Aspergillus niger*, *Aspergillus flavus*, Fermentasi keadaan pepejal
1.0 Introduction

Malaysia is abundant with lignocellulosic agroindustrial residues and one of them is sago starch processing industries (Vikineswary et al., 2005). Sarawak, located in East Malaysia is the largest sago-growing areas and now is the world’s biggest exporter of sago, exporting about 25,000-40,000 tons of sago products each year (Singhal et al., 2008). As stated by Awg-Adeni et al. (2012), sago starch processing mill that produced sago hampas residues are mixed together with wastewater are being washed off into nearby streams or accumulated in factory’s compound. Wastes that have been insufficiently disposed off may lead to serious environmental problems. Sago starch processing caused pollution problems that are more social and economical in nature that technological (Awg-Adeni et al., 2010). In addition, sago wastewater contravened the standard limit discharge enacted in the Environmental Quality Act, 1974, where the wastewater shows high organic material (‘hampas’), chemical oxygen demand (COD) and biological oxygen demand (BOD).

According to Kadarmoidheen et al. (2012), environmental pollution has been recognized as a worldwide threat to public health has given rise to a new massive industry for environmental restoration. For both economic and ecological reasons, decomposition or biological degradation has become an increasingly popular option for the treatment of agricultural, industrial, organic and toxic waste. By degrading the waste, its volume and quantity can be greatly reduced and also will helps in preventing environmental problems. Moreover, there are many microorganisms that have the ability to degrade and utilized the cellulose molecules as carbon and energy sources (Kadarmoidheen et al., 2012).
In this study, the biodegradation of sago *hampas* was done via solid state fermentation (SSF). *Aspergillus niger* and *Aspergillus flavus* were used in this study. According to Siddiqui *et al.* (2013), the capability of *Aspergillus* species to utilize every type of substrates is due to the variety of enzymes that they produced. Filamentous fungi are the best studied for solid state fermentation due to their hyphal growth which able to grow on the surface of the substrate particles and also penetrate through them (Toor & Ilyas, 2011). Al-Mahdi *et al.* (2012) stated that several microorganisms can make used the major components such as cellulose, starch, lignin and pectin both as a source of energy for growth and as carbon source for cell biomass synthesis which then producing enzymes and other high commercial value products.

The main objective of this project is to perform the degradation of sago *hampas* by *Aspergillus* spp. which are *Aspergillus niger* and *Aspergillus flavus*, via solid state fermentation. In addition, the productions of enzymes during solid state fermentation were determined. Therefore the specific objectives are to:

i. Perform the degradation of sago *hampas* by *Aspergillus* spp.

ii. Carry out solid state fermentation by *Aspergillus* spp. using sago *hampas* as substrate.

iii. Determine the presence of amylase and cellulase enzymes in the biodegradation sago *hampas*. 

3
2.0 Literature review

2.1 Sago hampas

Sago palm (Metroxylon sasu) as shown in Figure 1 is from genus Metroxylon and belongs to Palmae family (Awg-Adeni et al., 2010). According to Singhal et al. (2007), Malaysia is one of the leading world sago producers where sago is grown for the sago starch production or conversion to ethanol and animal feed. Sago palm is produced commercially in Sarawak, East Malaysia, has been recognized as the largest sago-growing areas and now is the world’s biggest exporter of sago.

Singhal et al. (2007) mentioned that the main carbohydrate source in Malaysia is sago starch and it has a low production cost and higher yields as compared to other starches. The pith of sago palm generated a starchy lignocellulosic by-product known as sago hampas after extraction process (Awg-Adeni et al., 2012). In Malaysia, about 50-110 t of sago hampas are produced daily in Sibu and Mukah Division and the amount of hampas released from the sago processing factory depends mostly on the extraction process quality (Awg-Adeni et al., 2010).

Sago hampas as shown in Figure 2 is mainly comprised of starch where on dry weight basis it contains 58% starch, 23% cellulose, 9.2% hemicelluloses and 4% lignin. On top of that, the hydrolyzation of polysaccharides of starch, cellulose and hemicelluloses into simple sugars can be utilized as a carbon source for fermentation process by microorganisms (Jenol et al., 2014).
2.2 Fungi

Fungi play a major role in the biodegradation of organic compounds (Rivilla et al., 2009). As a primary decomposer, it is crucial for fungi to decompose leaves, dead plants and other plant lignocellulosic biomass (Rittmann & McCarty, 2001). Fungi obtained sufficient carbon and nitrogen for survival by attacking lignocelluloses (Evans & Hedger, 2001). Fungi can grow on solid substrates and usually grown in the laboratory on defined media containing sugars such as glucose and sucrose or even on polymers such as cellulose (Cohen & Hadar, 2001). Furthermore, fungi can break down various polymers by secreting extracellular enzymes, which then reabsorbed by the fungal colony.
2.2.1 *Aspergillus* species

The genus *Aspergillus* has been studied as a model organism for fungal enzyme production (Subramaniyam & Vimala, 2012). Several enzymes of industrial importance have been extracted from these fungi, however, only few enzymes are manufactured at large scale and most of them are extracellular hydrolytic enzymes that have the ability to degrade naturally occurring polymers (Toor & Ilyas, 2014). According to Singh and Gupta (2014), among bacteria, *Bacillus* sp., and among fungi, *Aspergillus* sp. are widely reported as a robust producers of amylase. In addition, many species have been studied including *A. terreus*, *A. niger*, *A. fischeri* and *A. niveus* thereby making *Aspergillus* sp. to be known as a good cellulase producers (Danmek *et al.*, 2014).

2.2.2 *Aspergillus niger*

The largest fungal source of enzymes by far is *Aspergillus niger* (Subramaniyam & Vimala, 2012). *A. niger* as shown in Figure 3 is a filamentous fungus which grows aerobically on organic matter and usually found in soil, in compost and on decaying plant material (Schuster *et al.*, 2002). Junior *et al.* (2014) mentioned that *A. niger* is treasured in industry due to its high fermentation capacity, elevated levels of proteins secreted and the variety of enzymes produced for diverse applications which includes a combination of enzymes for plant cell wall polysaccharides degradation.
2.2.3 *Aspergillus flavus*

*Aspergillus flavus* as shown in Figure 4 is a fungus that turns into saprophytic mode when acquire resources for growth thus it is expected that *A. flavus* is able to produce a large array of enzymes to help in the degradation of complex substrates (Mellon *et al.*, 2007). A study by Bhardwaj *et al.* (2011) has proven that *A. flavus* is the best amylase producer.
2.3 Solid state fermentation

Solid state fermentation (SSF) is a fermentation process that takes place in the absence or near absence of free water (Toor & Ilyas, 2014). SSF is effective in the production of enzyme and is preferred when enzymes have to extract from fungi as it requires lesser water potential (Subramaniyam & Vimala, 2012). According to Cohen and Hadar (2001), in the absence of free water, only filamentous fungi can grow to a significant extent even though many microorganisms are capable to grow on solid substrates.

On top of that, fungal growth under SSF conditions is advantageous compared with submerged culture because less humidity is needed and the transfer of oxygen is much more efficient. Three classes among the filamentous fungi have gained practical importance in SSF: the Phycomycetes such as the genera *Mucor* and *Rhizopus*, the Ascomycetes with the genera *Aspergillus* and *Penicillium*, and the Basidiomycetes, especially the white rot fungi.

Cohen and Hadar (2001) mentioned that, there are five main purposes of the SSF process of filamentous fungi on agricultural wastes:

i. The degradation of cellulose and starch is to produce protein-rich animal feed.

ii. The degradation of lignin in lignocelluloses to give cellulose access to the cellulose for ruminant feed, saccharides production, feedstock for ethanol and the production of chemicals.

iii. The degradation of organic matter for the production of pure hydrolytic enzymes.

iv. The production of specific biochemical such as organic acids and saccharides.

v. The bioconversion of mixed organic wastes into stable organic product through composting processes.
2.4 Enzymes

Enzymes have very wide applications and have been considered as powerful tools in preserving the environment (Thakur et al., 2014). An increasing number of enzymes can be produced affordably because of the improved understanding of production biochemistry such as the fermentation processes and recovery methods. Nowadays, many microorganisms have been harvested from soil, fruits and vegetable and enzymes present in them actively engage in breaking down starch substrates into its simple forms (Geetha et al., 2011). The degradation and transformation of plant-cell wall polysaccharides which involved microbial enzymes have found many biotechnological applications.

2.4.1 Cellulase

Nowadays, the ability of cellulase enzyme to degrade cellulose are widely reported (Oyeleke et al., 2012). According to Sharada et al. (2013), cellulase can catalyze the hydrolysis of cellulose and related oligosaccharide derivatives thus it has been considered a potential tool for industrial saccharification of cellulosic biomass. In addition, when produced by SSF in comparison to submerged fermentation (SmF) enzyme production, the enzyme activities were increased about 30-80%.

The major obstacle to the exploitation of cellulase is that the cost production is high, including other factors such as complexity of cellulose structure, the type and source of cellulose employed for production and cellulases production by cellulolytic organisms is in low amounts. Nonetheless, the cost may be brought down by multifaceted approaches which include the use of cheap lignocellulosi substrates and the use of cost efficient fermentation strategies such as solid state fermentation (Sharada et al., 2013).
2.4.2 Amylase

El-Safey and Ammar (2004) mentioned that in the present day biotechnology, amylases are among the most important enzymes. These hydrolytic enzymes can be found in nature, animals, microorganisms and plants. According to Singh and Gupta (2014), in the present day amylases are also being looked for environment management through treatment of starch processing wastewater. *Aspergillus* sp. are considered to be vigorous producers of amylase, however, only few reports are available on *Aspergillus flavus* producing amylases.

Geetha *et al.* (2011) stated that amylolytic microorganisms play an important role in most of the food industries where enzymes present in them actively participate in the breakdown of starch substrates into its simple forms. Furthermore, starch which is a reserve source of glucose in plants and easily hydrolyzed by amylases produced by almost all living organisms.

2.5 Phenol-sulfuric acid assay

Nielsen (2015) stated that a simple rapid colorimetric method to determine total carbohydrates in a sample is the phenol-sulfuric acid method. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo- and polysaccharides. Concentrated sulfuric acid is used in this method to break down any polysaccharides, oligosaccharides and disaccharides to monosaccharides. In this method, the basic principle is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid will produce furfural derivatives. Then, further reaction between furfural derivatives and phenol would develops detectible color (Albalamesh *et al.*, 2013).
3.0 Materials and Methods

3.1 Fungi

The stock culture of *A. niger* and *A. flavus* were obtained from Molecular Genetic Laboratory Fungal Collection.

3.2 Inoculum preparation

*A. niger* and *A. flavus* were both maintained on Potato Dextrose Agar (PDA). As for the regular subculturing, the fungi were grown at room temperature for 7 days and stored at 4°C until further use. Approximately 5 mm (in diameter) fungal plugs were used as inoculums for fermentation process.

3.3 Substrate preparation

For preparation of substrate, sago *hampas* was blended to fine particles, sieved, autoclaved and stored in a closed beaker. Then, the substrate was ready to be used in SSF.

3.4 Solid state fermentation (SSF)

SSF was carried out by placing 5 grams of autoclaved sago *hampas* into a sterile 250 mL Erlenmeyer flask. Three plugs of fungus were inoculated onto the sago hampas within the Erlenmeyer flask. Mineral salt media was prepared and added to the flask. To perform the degradation of sago *hampas* using *Aspergillus* spp., one control set and three experimental sets were set up consisting of different fungal strains and combination of the *Aspergillus* consortia as inoculums.
All of experiments were done in triplicate except for control set. As described in the Table 1, each set were inoculated with different microorganisms. Each fermentation flask was then incubated at room temperature for a period of 10 days. All of the flasks used for fermentation were weighed before and after the initiation of fermentation.

Table 1: Experimental set with inoculation of different strain and combination of microorganisms.

<table>
<thead>
<tr>
<th>Experimental set</th>
<th>Aspergillus niger</th>
<th>Aspergillus flavus</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>✓</td>
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<tr>
<td>2</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>3</td>
<td>✓</td>
<td>✓</td>
</tr>
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3.5 Enzyme extraction

For the extraction process, the entire fermented sago *hampas* in the flask was mixed with 50 mL of 0.1 M sodium acetate buffer with pH 5.5. The mixture was shaken at room temperature at 150 rpm for 30 minutes. The slurry was filtered through muslin cloth and the filtrate was centrifuged at 10,000 rpm for 8 minutes at 4 °C (Chandra *et al.*, 2007). The clear filtrate obtained also known as the crude enzyme was used for enzyme assay.
3.6 Enzyme assays

Each of the samples was analyzed for total cellulase activity using filter paper (FPase) assay and amylase activity using starch soluble. A standard calibration curve was constructed by using glucose (refer Appendix D).

3.6.1 Cellulase enzyme assay

Filter paper assay was used in order to estimate the total cellulase activity of *Aspergillus* spp. that were grown and used as inoculums in solid state fermentation of sago *hampas*. This method was based on the method as described by Chandra *et al.* (2007), with slight modification of the amount crude enzymes used. A volume of 0.5 mL sodium acetate buffer with pH 5 was added into test tube having a cap followed by 0.5 mL of crude enzyme sample. Whatman no. 1 filter paper strip of dimension 1.0×6cm (50 mg) was placed into each assay tube for the FPase enzyme activity. The reaction was incubated for 10 minutes at room temperature and the reaction was stopped by adding 1 mL of 3, 5-dinitrosalicylic acid (DNS) solution and boiled for 10 minutes. After cool down, 1 mL of Rochelle salt (40 % sodium potassium tartarate) was added. The reaction mixture was measured against a reagent blank at 540 nm in a spectrophotometer.

One unit of enzyme activity is defined as the amount of enzyme required to release 1 milligram (mg) reducing sugar per minute.

3.6.2 Amylase enzyme assay

Amylase enzyme activity was determined by measuring the reducing sugar liberated from soluble starch substrate in the reaction mixture using the methods described in Geetha *et al.* (2011), with slight modification of the amount crude enzymes used. A volume of 0.5
mL of 1 % (w/v) soluble starch was mixed with 0.5 mL of crude enzyme. The mixture was then incubated at room temperature for 10 minutes and the reaction was stopped by addition of 1 mL of DNS. The tube was boiled for 10 minutes and after that, 1 mL of 40 % Rochelle salt was added. The reducing sugar released by enzymatic hydrolysis of starch was measured at 540 nm against a blank using spectrophotometer.

One unit of enzyme activity is defined as the amount of enzyme required to release 1 milligram(mg) reducing sugar per minute.

3.7 Determination of enzyme activity

Cellulase and amylase activity was calculated as follows:

\[
\text{Enzyme activity} = \frac{\text{Sugar concentration} \times \text{Total assay volume} \times \text{Dilution factor}}{\text{Enzyme volume} \times \text{minute of incubation}}
\]

3.8 Protein assay

The protein concentration of the crude enzyme was measured according to Bradford (1976) method and bovine serum albumin (BSA) as standard. Protein concentration was measured using spectrophotometer at 595 nm. The mg of protein was estimated based on BSA as standard curve (Appendix E).
3.9 Phenol-sulfuric acid assay

This method was based on the method as described by Albalasmeh et al. (2013), with slight modification. A 0.2 mL aliquot of carbohydrate (sample) solution and 0.2 mL of 5 % aqueous solution of phenol were mixed together in a test tube. Next, 1 mL of concentrated sulfuric acid was added quickly to the mixture. The mixture was diluted with the ratio of 1 to 5 dilutions, by adding 5.6 mL of sterile distilled water and placed for 10 minutes for color development. The light was recorded on spectrophotometer at 490 nm. Reference solutions were prepared in identical manner as above by replacing the 0.2 mL aliquot of carbohydrate with sterile distilled water. A standard calibration curve was formed by using glucose (Appendix F).

3.10 Percentage of degradation

By using given formula, percentage of degradation of sago hampas was calculated on the basis of sago hampas dry weight before and after fermentation.

\[
\text{Degradation (\%)} = \frac{X-Y}{X} \times 100 \%
\]

where: 

\(X\) – Weight of sago hampas before fermentation

\(Y\) – Weight of sago hampas after fermentation
4.0 Results and Discussion

4.1 Production of enzymes via SSF

*Aspergillus niger* and *Aspergillus flavus* were cultured on Potato Dextrose Agar (PDA) for 7 days before they can be used as inoculums. PDA is a recommended media to support the growth of fungi and it allows fungi to express their secondary metabolic capability. Current study was conducted under the solid state fermentation. This is due to the minimal moisture content in the solid state fermentation which highly decreased the risks of contamination (Toor & Ilyas, 2014). Moreover, Suganthi et al. (2011) mentioned that solid state fermentation holds excellent potentials for the enzymes production.

Before extracting the enzyme, 50 mL of acetate buffer was added in order to release the enzyme from inside the cell. The reducing sugar released during enzyme activity was determined by using DNS method and using glucose as standard. The results on optical density (OD) were recorded as presented in appendices part.

4.2 Cellulase and Amylase enzymes activity

Cellulases comprise a complex of enzymes which involved in the natural degradation of the major polysaccharide of plant cells, cellulose (Singh et al., 2009). The enzymatic complex works in the conversion of cellulose to oligosaccharides and glucose. Gomathi et al. (2012) quoted that the induction and activity of cellulase depend on the nature of substrate as cellulases are inducible enzymes. Ladokun and Adejuwon (2011) mentioned that enzyme amylase can break starch down into sugar. One of the common contaminants of starchy foods is *Aspergillus species*. This species can also grow in or on many plants and trees.