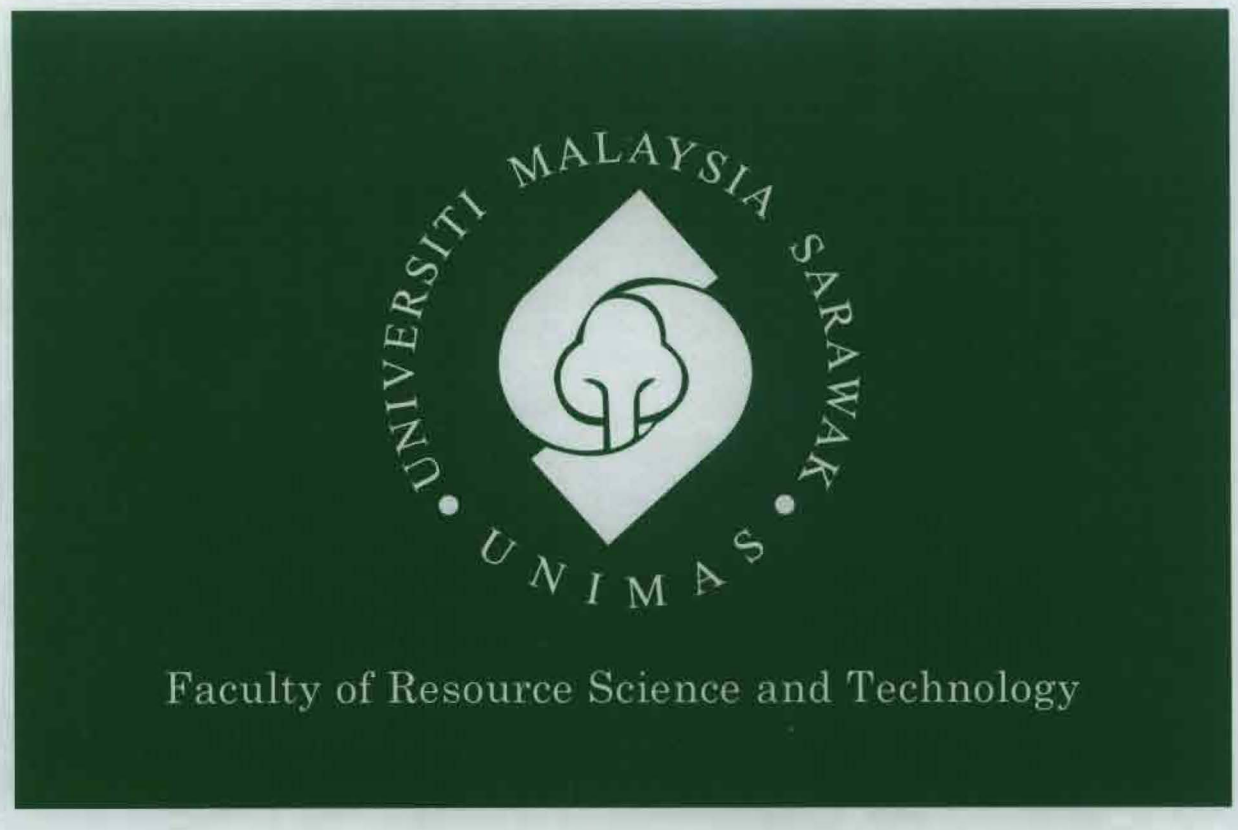

Production of Crude Xylanase from *Aspergillus niger* under Solid State Fermentation (SSF) by Using Different Agro Wastes as Substrates

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Bachelor of Science with Honours
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Production of Crude Xylanase from *Aspergillus niger* under Solid State Fermentation by Using Different Agro Wastes as Substrates

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This project is submitted in partial fulfilment of the Final Year Project (STF 3015)

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Declaration

I hereby declare that the study entitled 'Production of Crude Xylanase from *Aspergillus niger* under Solid State Fermentation by Using Different Agro Wastes as Substrates' submitted to Faculty of Resource Science and Technology, Universiti Malaysia Sarawak is my original work and that all the sources that I have cited have been acknowledge in the references section. It has been submitted and shall not be submitted in any form to any institution or other university.

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List of Abbreviations

BSA	Bovine serum albumin
DNS	Dinitrosalicylic
g	Gram
GRAS	Generally recognised as safe
min	Minutes
ml	Mililiter
nm	Nanometer
OD	Optical Density
OPEFB	Oil Palm Empty Fruit Bunches
PDA	Potato dextrose agar
pH	Partial hydrogen
rpm	Rotation per minutes
SmF	Submerged fermentation
SSF	Solid state fermentation
U	Amount of enzyme release 1 μmol/min xylose/minutes

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Abstract

Agro waste is a highly potential material that can be used as a substrate in solid state fermentation (SSF) for the production of enzyme. The problem caused by agro wastes can be reduced by manipulating them to produce xylanase which is one type of enzyme that provide numerous applications to industries. This study is focused on the production of crude xylanase from *Aspergillus niger* by using different types of agro wastes as substrates namely pineapple peels, rice husks and oil palm empty fruit bunches through SSF. The SSF parameters such as incubation period, initial moisture content of substrate and incubation temperature that affecting SSF to produce maximum enzyme production in term of enzyme activity were studied. Based on the results, it has found that the best substrate that produced the highest enzyme activity of 0.404 U/ml was pineapple peel with incubation for 8 days at 40 °C by using initial moisture content of 70%.

Key words: Xylanase, *Aspergillus niger,* solid state fermentation, agro wastes

Abstrak

Sisa pertanian merupakan salah satu bahan yang berpotensi tinggi untuk digunakan sebagai substrat bagi menghasilkan enzim. Masalah yang melibatkan sisa pertanian dapat dikurangkan dengan memanipulasikan bahan buangan tersebut untuk menghasilkan xylanase iaitu salah satu enzim komersial yang banyak diaplikasikan dalam pelbagai industri. Kajian ini memfokuskan kepada penghasilan enzim xylanase mentah daripada Aspergillus niger *dengan menggunakan kulit nanas, sekam padi dan tandan buah kosong kelapa sawit melalui kaedah fermentasi keadaan pepejal. Parameter fermentasi keadaan pepejal seperti tempoh masa inkubasi, kandungan kelembapan awal substrat dan suhu inkubasi yang mempengaruhi fermentasi keadaan pepejal untuk penghasilan enzim secara maksimum telah dikaji. Berdasarkan hasil kajian didapati substrat terbaik yang menghasilkan xylanase mentah dari segi enzim aktiviti tertinggi iaitu sebanyak 0.404 U/ml adalah kulit nanas dengan tempoh inkubasi selama 8 hari pada suhu 40°C dengan mengunakan kandungan kelembapan awal substrat sebanyak 70%.*

***Kata kunci*:** Xylanase, Aspergillus niger, fermentasi keadaan pepejal, sisa pertanian

1. Introduction

Malaysia is a country that is well-known for their agricultural activities. Paddy, palm oil and pineapple are some of the commercial crops found in Malaysia and contribute for most of the economic growth (Department of Statistic Malaysia, 2013). The problems associated with these crops are their massive wastes after being processed. Every year, Malaysia produces approximately 5 million tons of agricultural wastes (Pang *et al.*, 2006). The agro wastes generally accumulate in the environment and poses tremendous pollution problems (Okafor *et al.*, 2007). The most abundant organic compound set up in nature is plant cell wall polysaccharides which are found in the plant cell wall. Hemicelluloses are the second organic structure in plant cell wall and more heterogeneous compared to cellulose. Xylan is the major hemicellulose polymer in cereals and hardwood, representing up to 30 to 35 % of the total dry mass (Maciel *et al.*, 2008).

However, these problems can be reduced by development of solid state fermentation (SSF). Solid state fermentation offers advantages over submerged fermentation (SmF), particularly for fungal cultures. SSF has considerable economical potential in producing products for the food, feed, pharmaceutical and agricultural industries (Pandey *et al.*, 2000). One of the applications of SSF is the conversion of agro wastes into value-added products by utilisation of microorganism without or nearly absence of free flowing water to produce secondary metabolite products. *Aspergillus* species including *Aspergillus niger* are filamentous fungi usually used in SSF to produce enzyme including xylanase (Shah & Madamwar, 2006). Xylanase is an enzyme for degradation of xylan found in hemicelluloses of lignocellulosic materials. Xylanase can be applied in various industrials application such as biobleaching industries, food industries and for animal nutrient improvement (Motta *et al.*, 2013). Xylanases are fast becoming a major group of industrial enzymes finding significant application in paper and pulp

industry. Xylanase is important to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent. The utilisation of agro waste as substrate in SSF give some advantages in reducing the enzyme production cost instead of using expensive raw materials. Also, it can increase the concerns among citizen on the environmental issues and the recycling of waste disposal (Maciel *et al.*, 2008).

The problem statement of this study is there is high demand of xylanase in industry applications but the production cost for xylanase is too high. The production of xylanase from local agro wastes is still little known, thus this study was conducted in attempt to produce crude xylanase from *A. niger* via SSF by using different agro wastes as the substrates which are pineapple peels, rice husks and OPEFB. The specific objectives of this study are:

i. To produce xylanase from *A. niger* by using different agricultural wastes via SSF.

ii. To determine optimal parameters of SSF for the production of highest crude xylanase activity and specific enzyme activity.

iii. To identify the type of agro wastes that produces the highest crude xylanase activity.

2. Literature review

2.1 Xylanase

Xylanases are enzyme responsible for degradation of xylan. Xylanase is classified as glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which break down 1, 4-D-xylosidic linkages in xylan (Collins *et al.*, 2005). Endo-1, 4-xylanase (EC 3.2.1.8) and xylan-1, 4-xylosidase (EC 3.2.1.37), or also known as exoxylanase, are enzymes capable of cleaving the main-chain glycosyl groups (Maciel *et al.*, 2008). Xylan is a heterogeneous polysaccharide that linked with glucoronic acid, arabinofuranose, and acetyl group that connected to the D-xylose backbone as shown in **Figure 1**. Xylan consists of a b-1, 4-linked D-xylose backbone and can be substituted with different side-groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glucuronic acid residues (Maciel *et al.*, 2008).

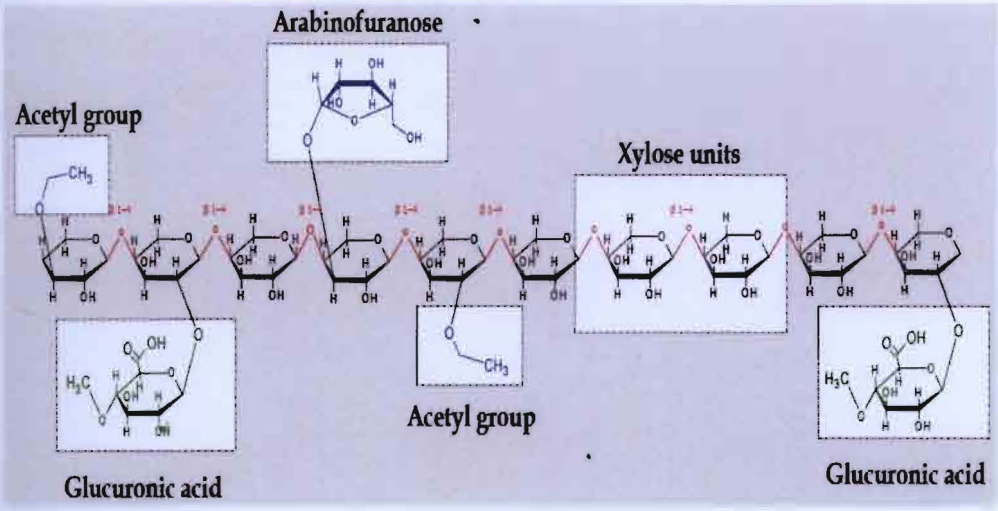

Figure 1: Representation of a hemicellulose structure formed by a xylan backbone.

(*http://www.intechopen.com/books/sustainable-degradation-of-lignocellulosic-biomass*).

Xylan can be found between interface of lignin and celullose which is crucial for fibre cohesion and to maintain plant cell wall integrity (Beg *et al.*, 2001). The main xylanolytic

enzymes are endo-β xylanase and β xylosidase which attack the main chain of xylan and hydrolyse xylooligosaccharide into D-xylose respectively (Kanimozhi & Nagalakshmi, 2014).

Xylanases are extracellular enzymes produced by microorganisms such as saprophytic and phytopathogenous bacteria, mycorrhizic fungi as well as some yeasts. Based on the previous study, it has shown that solid state fermentation (SSF) using various agro wastes can be applied to produce xylanase with low production cost for industrial applications (Kanimozhi & Nagalakshmi, 2014). For fungal xylanase, the optimum pH for xylan hydrolysis is around 5 and normally stable at pH between 3 to 5 and have initial cultivation pH lower than pH 7 (Subramaniyan & Prema, 2002).

Enzyme plays a central role in many manufacturing processes. Xylanase has becoming one of the important enzymes in pulp and paper industries as it facilitates the breakdown of xylan to release lignin from paper pulp and reduces the usage of chlorine as the bleaching agent (Subramaniyan & Prema, 2002). Potential applications of xylanase also include bioconversion of lignocellulosic material and agro wastes to fermentative products. Xylanase has been used in various industrial applications to manufacture varieties of products as shown in **Table 1.**

Table 1: Applications of xylanase in various industrial fields.

Fields	Applications	References
Animal feeding	Enhancement nutritional value and help digestion in ruminants	Motta *et al.* (2013)
Baking industry	Improve flour quality by enhancing the raise of bread when baked	Mohd Nor (2006)
Biobleaching of wood pulp	Get rid of residual xylan during dissolving pulp process	Madlala *et al.* (2001)
Bioenergy industry	Boost fermentation efficiency and reduce the cost	Motta *et al.* (2013)
Food industry	Produce xylitol which is important as sweeten in chewing gum, ice cream and candy	Kanimozhi & Nagalakshmi (2014)

2.2 *Aspergillus niger*

Aspergillus species are group of mold which are ubiquitous in the nature environment. This molds also known as filamentous fungi. These filamentous fungi are also saprophytic, aerobic fungi that develop on dead or decaying organic matter. Most filamentous fungi are growing aerobically on organic matter. Based on U.S. Environmental Protection Agency, *Aspergillus* is widely distributed geographically, and also has been observed in a broad range of habitats. The growth of these fungi is depending on the influence of temperature, water activity and pH. Filamentous fungi have better growth at low water activities compared to yeasts and bacteria (Bhardwaj *et al.*, 2011). Thus, making the filamentous fungi preferred used in SSF compared to other microorganism.

Aspergillus niger is a haploid aerobic filamentous fungi and it is a very important microorganism used in the biological field. *A. niger* is commonly found in mesophilic environments such as soil, plants, and enclosed air. *A. niger* is a xerophilic fungi which is a

type of mold that does not need water for growth yet capable to thrive in humid environments and besides it is also a thermo-tolerant organism that can tolerate high temperatures (Okafor *et al.*, 2007).

A. niger consists of mycelial, or threadlike, hyphae that are separated by a septum and transparent and it produce colonies which contained white or yellow felt covered by dark asexually produced fungal spores called conidiophores as shown in **Figure 2** (Souza *et al.*, 2011). *A. niger* genomes consists of 14,600 genes and about 200 genes are involved in polysaccharide degradation (Souza *et al.*, 2011).

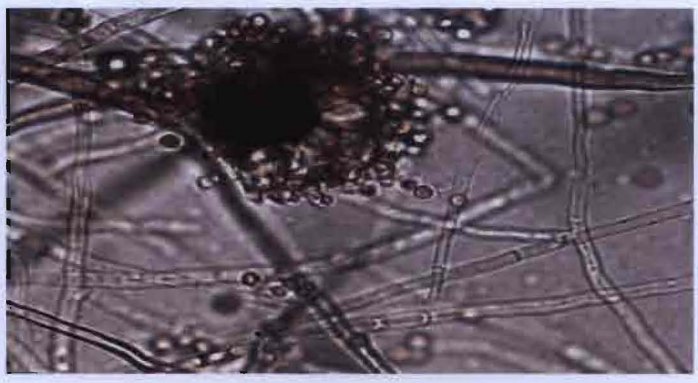

Figure 2: Microscopic view of *A. niger* using 400X magnification. (*http://www.napavalley.edu/people/srose/Pages/Biol220.aspx*).

There are many of the enzymes produced by *A. niger* are considered generally recognised as safe (GRAS) by the United States Food and Drug (Motta *et al.*, 2013). One of the applications of *A. niger* involve the production of citric acid and industrial enzymes such as amylase, protease, and lipase (Souza *et al.*, 2011). In addition to producing extracellular enzymes and citric acid, *A. niger* is used for waste management and biotransformation. Recent study has shown that *A. niger* is capable to produce xylanase by using agro waste as substrate which can reduced the environmental problem caused by accumulation of the agro waste (Okafor *et al*, 2007). Others studies have shown that filamentous fungi including *A. niger* have been widely used to produce hydrolytic enzymes

for industrial applications, including xylanase, which the level of enzyme released by fungi are generally much higher than those from yeast and bacteria. On an industrial scale, xylanase is produced mainly by *Aspergillus* and *Trichoderma* spp. (Maciel *et al.*, 2008).

2.3 Solid State Fermentation (SSF)

SSF caught many attentions because it has several advantages compared to submerged fermentation (SmF). SSF offers higher fermentation productivity, higher product's end-concentration and stability, lower catabolic repression, microorganism cultivation is specific for insoluble substrate, and have lower degree of contamination due to low water activity used (Bhardwaj *et al.*, 2011). Although a number of xylanase productions were performed using submerged systems but solid state fermentation was found to be more economical primarily owing to the low-cost and accessibility of agricultural wastes (Pandey, 2003).

SSF is suitable for processing agro industrial wastes but requires some optimal parameters such as the selection of appropriate microorganism and substrate, parameters used to optimise the process and also extraction and purification of the yield (Pandey, 2003). Only fungi and yeast are suitable for SSF instead of others microorganism based on the theoretical principle of these two microorganisms on water activity whereas bacteria have been considered inappropriate microorganism (Chinn *et al.*, 2007).

There are several parameters influencing SSF process such as initial moisture content of substrate, incubation temperature, incubation period, pH stability, inoculum sizes and substrate amount (Pandey, 2003). The moisture content can be accustomed by adding the moistening agent such as sterile distilled water to give the moisture content ranging from 65 to 85% (v/w) which is the best range of moisture content to optimise the SSF process (Pang *et al.*, 2006). Based on the study done previously, 45°C is the best

incubation temperature for the high xylanase activity when using wheat bran as substrate (Kavya & Padmavathi, 2009). The most suitable incubation period to produce high xylanase activity is for 6 days and after 6 days, the xylanase activity will decrease (Kavya & Padmavathi, 2009).

Fungal growth and secondary metabolite production in SSF are significantly affected by temperature and heat transmission processes in the substrate bed. During SSF a large amount of heat is generated, which is related to the metabolic activities of the microorganism. The heat transfer in and out of an SSF system is closely connected with microbial metabolic activity and aeration of the fermentation system. High temperature influenced fungal germination, metabolites development and sporulation (Raghavarou *et al.*, 2003).

2.4 Pineapple Peels as Substrates

Malaysia is popular as one of the main pineapple-producing countries of the world. According to statistic published by Malaysian Pineapple Industry Board (n.d.), Malaysia produced 69,607 ton metric in 2007 and it increased up to 156,111 ton metric in 2008. The increasing of pineapple-product industries can be the indication for the increase of pineapple waste in this country.

Lignocellulose is the main component of woody plants and non woody plants that represents a major source of renewable organic matter. Agro wastes will act as solid inert matrix for the fungi, providing nutrients as well as provide support system. Pineapple from species *Ananas comosus* is one of the commercialised plants available throughout Malaysia. Pineapple wastes consist of core, peels and crown. It is reported that 40 to 80% of pineapple wastes accumulates in environment lead to high number of organic materials and suspended solid (Bankoffi & Han, 1990). Pineapple peels consists of many

polysaccharides fibre-rich which are cellulose, hemicellulose, pectin and lignin (Huang *et al.*, 2011). The composition of pineapple peels are 40.55% cellulose, 28.69% hemicelluloses and 10.01% lignin (Pardo, 2014).

2.5 Rice Husks as Substrate

Rice husks are one of the wastes widely available in most rice producing country like Malaysia. Normally after the rice has been processed, the husks are been discarded by burning or dumped as wastes (Kumar *et al.*, 2012). The main polysaccharides found in the rice husks are 25 to 35% cellulose, 18 to 21% of hemicellulose and 26 to 31% of lignin (Masutti *et al.*, 2012).

2.6 Oil Palm Empty Fruit Bunches (OPEFB) as Substrate

Malaysia is the second largest palm oil producer. In the process of palm oil extraction, a large amount of oil palm empty fruit bunch (OPEFB) is generated as waste products. It is estimated that Malaysia produced about 7.7 million tons of OPEFB annually as wastes (Zwart, 2013). However, most of this waste is burnt as an alternative to energy source resulted in thermal and potential air pollutions when treated unsystematically (Rahman *et al.* 2006). There are about 59.7% of cellulose found in the OPEFB, 22.1% of hemicellulose and 18.1% of lignin (Geng, 2013).

3. Materials and Methods

3.1 Preparation of *Aspergillus niger*

Aspergillus niger was obtained from Molecular Genetics Laboratory, FRST, UNIMAS. The strain was sub-cultured on potato dextrose agar (PDA) with 50 mg/ml ampicillin and grew at room temperature for several days. Then, it was maintained at 4°C before used.

3.2 Preparation of substrates

Pineapple peels, rice husks and OPEFB were the substrate used in this study. Pineapple peels were collected from pineapple plantation in Kota Samarahan. The peels were washed and oven-dried at 60°C for 3 days. The dried pineapple peels was milled and sieved prior to use. Rice husk and OPEFB were obtained from Molecular Genetics Laboratory, FRST, UNIMAS. The rice husks were separated from rice weevils and further pre-treatment was not required because the supplied rice husks were already ground. OPEFB were ground into smaller size by using electrical blender. The substrates were dried in an oven in order to know the existing moisture content in the substrate (refer to **Appendix A** for the calculation).

3.3 Preparation of reagent

The reagent used in this project such as 3,5-dinitrosalicyclic acid (DNSA), 40% Rochelle's salt, 0.1M sodium acetate buffer with pH 5.8, 1% xylose solution, 1% xylan birchwood, bovine serum albumin (BSA), and Bradford's reagent were prepared by using standard procedure.

3.4 Solid state fermentation (SSF)

Cultivation of *Aspergillus niger* was performed in a 250 ml Erlenmeyer flask contained 5 g of solid substrate and sterile distilled water was added into the substrate to achieve 70% initial moisture content of the substrate (refer to **Appendix A** for the calculation). The substrate was then inoculated with the fungi using 3 plugs. Then, it was incubated at 30°C for 6 days in a static condition (Mrudula & Murugammal, 2011). The experiments were carried out in a duplicate.

3..5 Crude xylanase extraction

A volume of 0.1 M cold sodium acetate buffer with pH 5.8 was added to the SSF medium after SSF process has completed. Then, the flasks were shaken for 30 minutes for 120 rpm at room temperature. The solid biomass residues were separated from the suspension by filtration using muslin cloth. After that, the mixture was centrifuged at 6000 rpm for 20 minutes at 4°C. The supernatant was collected and filtered by using filter paper, twice. The supernatant was used as the source of crude enzyme (Kanimozhi & Nagalakshmi, 2014).

3.6 Xylanase activity assay and protein determination

Xylanase activity was measured using mixture inside test tube containing 0.25 ml crude enzyme and 0.25 ml xylan birchwood solution. For blank, the crude enzyme was substituted with sodium acetate buffer. The samples were incubated for 30 minutes at 50°C. Then, 0.5 ml of DNS reagent was added into the test tube The release reducing sugar will be determined by the 3, 5-dinitrosalicylic (DNS) acid method with xylose as standard (Miller, 1959). The colour was developed by boiling for 15 minutes in water bath. Rochell's salt was immediately added before the sample cooled. After the samples were cooled, the samples were measured at 550 nm by using spectrophotometer. One unit of

activity (U) releasing reducing sugar is defined as amount of enzyme required to liberate 1 µmol of xylose per minute (µmol/min) under the assay conditions (Maria *et al.*, 2006). Protein determination was examined by Bradford's method using bovine serum albumin (BSA) as standard (Guimaraes, 2013). For protein assay, 20 µl sodium acetate buffer, 20 µl of crude enzyme and 1 ml of Bradford's reagent were added in a cuvette and measured at 595 nm using spectrophotometer (Guimaraes, 2013).

3.7 Preparation of xylose as standard and bovine serum albumin (BSA) standard

Xylose standard was prepared in order to determine the concentration of sugar released by xylanase enzyme. The OD value for different known concentrations of xylose which were (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4) mg/ml was determined. BSA standard was prepared to determine the different protein concentration which were (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0) mg/ml. Refer to **Appendix E** for standard graph as reference.

3.8 Determination of xylanase activity and specific enzyme activity

Xylanase activity was determined by using formula of enzyme activity. Refer to **Appendix C** and **D** for enzyme activity and specific enzyme activity calculation.

Enzyme activity (U/ml):

$$\frac{\text{Reducing sugar released (mg) X total assay.volume (ml) X dilution factor}}{\text{Enzyme volume (ml) X Volume of sample in cuvette (ml) X minute of incubation (min)}}$$

Specific enzyme activity (U/mg):

$$\frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

3.9 Optimisation of SSF parameters

3.9.1 Effect of incubation period

The cultivation process in SSF was carried out in different incubation period to find the optimum condition for SSF. The experiments were carried out for 4, 6, 8 and 10 days by 48 hours intervals to check for enzyme activity.

3.9.2 Effect of initial moisture content

Range of initial moisture content of 60%, 65%, 70%, and 75% were studied to optimise the condition for SSF for maximum xylanase activity.

3.9.3 Effect of incubation temperature

Temperature is one of the parameters that determine the success of optimization system. Therefore, the effect of temperature on xylanases production by *Aspergillus niger* was examined at various temperature range of 30°C, 35°C, 40°C and 45°C

4. Results and Discussion

4.1 Production of xylanase by SSF

Aspergillus niger was sub-cultured on potato dextrose agar (PDA) that contain ampicillin to prevent any bacterial growth as shown in **Figure 3**. The media were maintained at 4°C prior to use. To inoculate the fungi into substrate, three plugs with the same size and same spore distribution were taken. It is important so that the distributions of spores in all the media are constant throughout the experiments.

Figure 3: Growth of *A. niger* cultured on PDA after 7 days

Xylanase was produced by culturing *A. niger* with different substrates which were pineapple peels, rice husks and OPEFB in different flasks using fixed incubation period, initial moisture content and incubation temperature which were 6 days, 70% and 30°C respectively. These parameters were taken because of the previous knowledge from other study that showed maximum xylanase activity (Kanimozhi & Nagalakshmi, 2014). For pineapple peels, 10.195 ml of ddH_2O was added to the substrate, 8.925 ml for rice husks and 8.705 ml for OPEFB to achieve 70% initial moisture content. The existed moisture content in the substrate was calculated by using dry basis formula in order to know how much water need to be added to achieve desired initial moisture content of the substrate as shown in **Appendix A**. Sodium acetate buffer with pH 5.8 was used in this study as