Optimisation of Solid-State Fermentation Parameters for the Xylanase Produced by *Aspergillus niger* using rice straw as the substrate

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
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Supervisor: Dr. Rosmawati binti Saat

Programme of Resource Biotechnology
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ABSTRACT

Rice straw is one of the most abundant lignocellulosic waste materials and a major agricultural by-product in Asia. Improper management of this agro-waste such as open field burning waste may become a serious threat to environment and health. Rice straw has been considered as a feasible medium for xylanase production under solid-state fermentation. This study focused on the optimization of SSF parameters for production of xylanase by *Aspergillus niger* utilizing rice straw as the substrate. The SSF parameters that were studied are initial moisture content of substrate, incubation temperature and period . Total enzyme activity was determined and amount of reducing sugar released was assessed through dinitrosalicylic acid method. The highest xylanase activity of 2.5352 ± 0.2667 U/ml was achieved at 70% (v/w) initial moisture content, 30°C of incubation temperature, and 4 days of incubation time.

Keywords: rice straw, *Aspergillus niger*, Solid-state fermentation, Xylanase.

ABSTRAK

Jerami padi adalah salah satu sisa buangan lignuselulosa terbanyak dan merupakan sisa agrikultur terbesar di Asia. Ketidakcekapan dalam pengurusan sisa pertanian ini seperti pembakaran secara terbuka boleh menjadi impak yang serius kepada alam sekitar dan kesihatan. Jerami padi telah dianggap sebagai medium yang berkesan untuk penghasilan xylanase melalui kaedah fermentasi keadaan pepejal. Kajian ini memfokuskan kepada optimasi parameter SSF untuk penghasilan xylanase oleh Aspergillus niger *menggunakan jerami padi sebagai substrat. Parameter SSF yang akan dikaji adalah kandungan pelembapan awal, suhu dan tempoh pengeraman. Jumlah aktiviti enzim telah dikenalpasti dan jumlah pengurangan gula dibebaskan telah dinilai melalui keadah asid dinitrosalisilik. Aktiviti xylanase tertinggi sebanyak 2.5352 ± 0.2667 U/ml telah dicapai pada 70% (v/w) kandungan awal substrat, suhu inkubasi 30°C dan 4 hari tempoh inkubasi.*

Kata kunci: jerami padi, Aspergillus niger, fermentasi keadaan pepejal, xylanase

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List of Abbreviations

A.niger	*Aspergillus niger*
SSF	Solid-state fermentation
SmF	Submerged fermentation
a_w	Water activity
FRST	Faculty Resource Science and Technology
UNIMAS	Universiti Malaysia Sarawak
PDA	Potato Dextrose Agar
DNS	Dinitrosalicylic acid
GRAS	Generally Regarded as Safe
GHSs	Greenhouse gas emissions
μm	Micrometre
U	unit
mg/ml	Milligrams per milliliter
DDW	Double distilled water
°C	Degree Celcius
EMC	Existing moisture content
FAO	Food and Agricultural Organization of United Nation
M	Molarity
ml	mililitre

NaOH	Sodium hydroxide
nm	nanometre
rpm	Revolution per minute
SD	Standard deviation
v/w	volume over weight
w/v	Weight over volume
μmol	micromolar

1.0 INTRODUCTION

Since Asia is the world's biggest rice producer and consumer, therefore rice is regarded as the world's second most important crop after wheat (Firdaus et al., 2020). According to The Observatory of Economic Complexity (2020), Malaysia exported roughly $48.2 million worth of rice in 2020, making it the 34th largest exporter of rice in the world**.** Other than exportation, rice is also demanded in huge quantity in Malaysia as it is the staple food and crucial diet in daily meal of Malaysian. The rice producing zones in Malaysia is located on the Malaysia Peninsular and on Borneo Island (*Food and Agriculture Organization of the United Nations* , n.d.). Kedah, Sarawak, Perak, and Kelantan are the major harvest are of rice (*Food and Agriculture Organization of the United Nations*, n.d.). The major problems associated with the production of rice is huge number of wastes after being processed. Based on the study done by Shafie (2015), the amount of total paddy residues produced in Malaysia are 3,324 million tonnes, consisting of 99.75% rice straw and 97.1% rice husk. Rice straw is one of the agriculture-by-product. Globally, the quantity of rice straw produced per year was about 570 million tonnes (Norazlina et al., 2013). Conventionally, rice straw was burned openly as a waste disposal approach by farmers. This practice resulted in the emission of a significant amount of greenhouse gases into the atmosphere that may affects the air quality, cause climatic changes, and deteriorates public health (AMK, 2020)

Rice straw is composed of 35-50% cellulose, 15-30% hemicellulose, 20-30% lignin and a slight amount of ash nitrogenous (Rosmiza et al., 2019). Hence, rice straw can be utilized as a substrate in the SSF process for inducing xylanase due to major portion of hemicellulose known as xylan (Zahari et al., 2016). Solid state fermentation (SSF) is defined as a fermentation process which microorganisms grow in an environment absent of free water, or with minimal content of free water (Soccol et al., 2017). SSF offers numerous advantages over submerged fermentation (SmF) such as potential to yield a higher quantity of enzyme at a lower cost and the ability to mimic the natural habitat of microorganism, especially, fungi (Ridder et al., 1998).

In addition, SSF utilizes lignocelluloses as a substrate to induce xylanase production by the fungi, thereby it is a cost-effective method and offer environmental advantages. Diverse species of fungi and bacteria can produce xylanase, but according to Knob et al. (2014), filamentous fungi are considered as more potent xylanase producers compared to bacteria or

yeast, thus, making it widely utilized as an enzyme producer. One of the most widely used fungi for commercial production of pharmaceuticals and enzymes is the *Aspergillus niger* (Bellaouchi et al., 2021). The capability of *A.niger* to synthesise a broad range of enzymes involved in the degradation of plant polysaccharides such as cellulose, xylan, xyloglucan, galactomannan, and pectin is well known (Bellaouchi et al., 2021). Xylanase is one of the enzymes that can be produced through SSF process, and it is demanded in substantial quantity for its various industrial applications and of its unique properties that allow it to withstand harsh industrial processing (Qiu et al., 2010). Xylanase is efficient to hydrolyse the xylosidic linkages in xylan (Ridder et al., 1998). It is a fundamental element of hemicelluloses and second most abundance in plant matter after cellulose (Ridder et al., 1998). The main industrial application of xylanase is as bleaching agent of wood kraft pulps (Knob et al., 2014). Hence, selection of potent microorganisms for xylanase synthesis is necessary, accompanied by optimization of SSF parameters to improve enzyme production (Bhardwaj et al., 2019).

Utilization of rice straw as the substrate in SSF fermentation to produce xylanase enzyme had been studied and it shows promising results . However, few studies have been done to optimize the SSF parameters such as initial moisture of substrate, incubation temperature and incubation period by using rice straw as the substrate. Optimal environmental conditions are necessary for the SSF to produce high enzyme activity as it stimulates maximum growth of the microorganism and improved production of the enzyme (Ridder et al., 1998). According to Park et al. (2002), the goal of basic research for industrial application is to optimize the fermentation medium and process to reduce the cost of enzyme production. The growing environments of the SSF includes several variables such as temperature (Ridder et al., 1998), moisture content (Smits et al., 1996), and the length of time for fermentation (Bailey et al., 1992).

Therefore, this research study focuses on optimisation of SSF parameters for the xylanase produced by *Aspergillus niger* using rice straw as the substrate. The SSF parameters that were being optimized are initial moisture content, incubation temperature and period of incubation. Subsequently, xylanase activity was determined followed by determination of reducing sugar released by dinitrosalicylic acid (DNS) method (Miller, 1959).

Objectives

Therefore, this research study mainly aims:

1. To determine the optimal initial moisture content, incubation temperature and incubation period for xylanase production by *A.niger* using rice straw as the SSF substrate.
2. To produce high xylanase activity using rice straw as the substrate in SSF.

2.0 LITERATURE REVIEW

2.1 Solid-State Fermentation

2.1.1 Solid-State Fermentation Process

Solid-state fermentation (SSF) is described as the fermentation's process which involved solid in the absence or minimal presence of free water; nonetheless, the substrate must have enough moisture to support the growth and metabolism of microorganism (Pandey, 2003). The solid matrix used in this process are either source of nutrients or a support impregnated by the proper nutrients which provide the product developments of the microorganism (Singhania et al., 2009).

Traditionally, industrially important enzymes have been produced using submerged fermentation (SmF), an approach that is simple to handle and allows for accurate and better monitoring of environmental variables such as temperature and pH (Mrudula & Murugammal, 2011). In SmF, microorganism is grown on soluble or insoluble substrate that are dissolved or submerged in liquid media (Kapoor et al., 2016). However, solid state fermentation technique has numerous advantages over SmF for the production of microbial enzymes, such as greater productivities and yield, lower operating costs, cheaper fermentation media by using agro industrial residues, greater oxygen distribution, fewer operational issues including simpler equipment and control systems (Ramos Sanchez, 2015). Besides that, between these two processes, solid state fermentation is more economical as the substrates that can be used are agricultural waste which is cheaper and abundantly available (Kheng et al., 2004). A study conducted by Nair et al. (2008), found that xylanase production in SSF is significantly higher compared submerged fermentation (SmF). In addition, research conducted by Ramos Sanchez (2015) stated that lipase production by SSF remained stable for 15 days, whereas lipase production in SmF began to decline on day 5.

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2.1.2 Solid-State Fermentation Parameters

There are physicochemical and biochemical parameters in SSF process which are pH, relative humidity pre-treatment and initial moisture of substrate, particle size, agitation and aeration, temperature of incubation, the size and age of inoculum, including supplementation of additional carbon source and inducers (Pandey, 2003). In addition, one of the main aspects in SSF parameters is the selection of a proper substrate as the non-soluble solid material as it will be the main source of nutrients and a physical support (Pandey, 2003). In order to maximize the enzyme yield using SSF, the environmental conditions need to be optimized to enhance maximum growth of the microorganism and elevate the production of the enzyme (Ridder et al., 1998).

Moisture content and temperature are the physiochemical factors that influence SFF process (Webb, 2017). Water activity (a_w) is the form to express quantitatively the water requirements of microorganism for their microbial activity (Webb, 2017). Based on the study done by Kavya and colleagues (2009), main factor for increasing xylanase production is initial moisture of the substrate. If the substrate is too moist, the porosity will decrease which cause oxygen cannot be penetrated, while if the substrate is low moisture level, organism will not growth (Pandey, 1992). As for temperature, the problem occurs during SSF process where the heat produced from microbial activity accumulated in the system (Webb, 2017). A study done by Seyis & Aksoz, (2003), showed that incubation temperature plays a crucial role for enzyme productivity. In addition, incubation duration is one of the SSF parameters that can be optimized to identify the time for the highest enzyme production. The incubation period is manipulated by the properties of the culture and also constructed on culture development rate and enzyme production (Uyar & Baysal, 2004). In basic research of industrial applications, the objective is to minimize the enzyme manufacturing costs via optimizing the fermentation medium and process (Park et al., 2002).

2.2 Xylanase

2.2.1 Properties of Xylanase

Xylanase is belonged to a class of enzyme called pentosanes which degrades the cell wall matrix of plant xylan into xylose (Hasan et al., 2016). Xylan is the polysaccharide with the most prominent structure in plant cells, and it is the most abundant polysaccharide in nature, comprising approximately one-third of the world's renewable organic sources (Hasan et al., 2016). Various living organism such as microorganism, protozoans, molluscs including rumen of higher animals formed xylanases (Beg et al., 2001). According to Collins et al. (2005) ,endohydrolysis of 1,4-β-D-xylosidic linkages in xylan are promoted by the xylanase which is a glycosidase (O-glycoside hydrolases, EC 3.2.1.x). The interaction between number of the main-chain and side-chain-cleaving enzyme activities are required to achieve a full cleavage of glycoside linkages located at heteroxylan backbone. One of the primary components of hemicelluloses located in plant cell wall is xylan and it is the second most prevalent polysaccharide after xylose (Kanimozhi & Nagalakshmi, 2014). Xylan has a linear back bone of -1,4- linked xyloses and constitute 15-30 percent of hardwoods, 30 percent of the cell wall material of annual plants and 7-10 percent of soft woods present in all terrain plants (Kanimozhi & Nagalakshmi, 2014).

2.2.2 Applications of Xylanase

According to Kanimozhi & Nagalakshmi (2014), solid state fermentation (SSF) can be employed in industrial application for producing xylanase with low production cost by using various agro wastes. The most functional utilisation of xylanase is prebleaching of kraft pulp where it can assist to limit the usage of hazardous chemicals in the subsequent treatment staged of kraft pulp (Beg et al.,2001). Moreover, application of xylanase can be found in the livestock feed, fabric, and food manufacturing industries also in the production of various useful products such as xylitol and ethanol (Nair et al., 2008). Maciel et al. (2008)

stated that, usage of xylanases in industries allow processes to be operated with minimum chemicals, under less harsh environments and with a lesser amount of disturbing side reactions. Both environment and society gain a lot of benefit from the use of xylanases in industry (Maciel et al., 2008).

2.3 *Aspergillus* species

2.3.1 Xylanase-producing Fungi

The genus *Aspergillus* comprise of organisms whose qualities are notable in pathological, agricultural, commercial, pharmacological, scientific, and cultural value and which play a key role in the degradation of organic substrate, especially plant material (Afzal et al., 2013). *Bacillus* and *Clostridium* are the bacterial species that have the potential to be used under SSF condition, while for fungal species, *Aspergillus*, *Trichoderma* and *Mucour* species are renown for the SSF process (Srivastava et al., 2019). The filamentous fungi are presumed to have the finest capacity to produce crucial industrial enzymes under SSF conditions (Srivastava et al., 2019). Knob et al. (2014) stated that utilization of fungi in bioprocesses has thrive in significance due to the production of various enzymes with assorted biochemical properties and outstanding potential for biotechnological application. Xylanase production is more potent by the usage of filamentous fungi as enzyme producers compared to bacteria or yeast (Knob et al., 2014). Both *Aspergillus* and *Trichoderma* spp. have been mainly used to produce xylanase on industrial scale (Maciel et al., 2008).

2.3.2 Aspergillus niger

Aspergillus niger can be discovered naturally in soil and litter, including in compost and on decaying plant material (Schuster et al., 2002). It is one type of filamentous fungus which grow aerobically on organic matter**.** Size of the fungus is in the range of between 900-1,600 μm in length which spherical conidia ranging from 3-5 μm (Phoebe, 2008). There are 14,600 genomes contained in *A. niger* and around 200 genes are associated in polysaccharide

degradation (de Souza et al., 2011). In recent years, *A. niger* have become an effective model fungus for basic studies due to annotated genome sequence, gene transfer systems and diversity of regulatory mutants (de Souza et al., 2011). Phoebe (2008) stated that *A. niger* can be easily cultivated in laboratory conditions, therefore making it one of the most extensively studied groups of fungus. Furthermore, according to Silva et al. (2011), *A. niger* species is widely used in biotechnological processes and exclusively has the GRAS status (Generally Regarded as Safe) status by the "Food and Drug Administration".

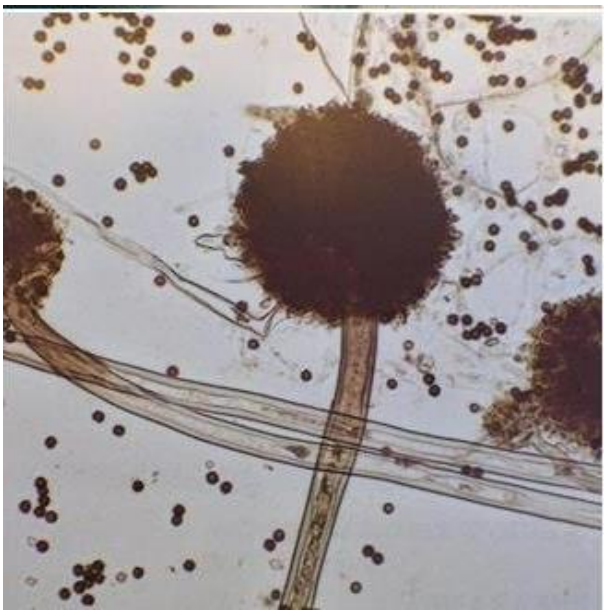

Figure 1: Microscopic view of *A.niger* (Abbas et al., 2021) .

Majority members of the *A. niger* group are significant makers of extracellular enzymes as well as essential plant cell wall hydrolyzing enzymes like xylanase (Okafor et al., 2010).Other than xylanase production, *A. niger* also are being utilised in the fermentation industries to generate organic acids such as gluconic acid and citric, and hydrolytic enzymes like amylases and lipases (Silva et al., 2011).Based on the study done by Nair et al. (2008), the comparison was done between several fungi species to analyse the top xylanase producer and the most prevalent species is *Aspergillus niger*. In addition, a study conducted by Gawande & Kamat (1999) found that strains of *Aspergillus terreus* and *A. niger* produces

xylanase with very low amount of cellulase activity which facilitate relatively simple enzyme purification systems. Besides that, in the xylanase recombinant production host, *A. niger* is among the fungi that dominate the scene (Knob et al., 2014).

2.4 Agro waste substrate

2.4.1 Rice straw as substrate

For the most part in Asia, rice (Oryza sativa) is the primary staple food, and the rice crops produce vast quantity of rice straw in the fields (Gautam et al., 2018). Rice straw is an agriculture residue. It is the dry stalk part of a paddy which can be collected after the grain or seed has been extracted (Norazlina et al., 2013). According to Food and Agriculture Organization of the United Nations (FAO), in 2021, Malaysia's average paddy production is estimated at about 2.5 million tonnes. Zahari et al. (2016) stated that, Malaysia's rice production to straw output ratio is constant from year to year which is 1:1 and it is being applied to approximate the amount of straw that will be acquired. On-farm open burning is the most preferable method used by most of the farmers to control the straw waste, however, it may contribute to several problems such as global climate change, effect on human health and greenhouse gas emissions (GHSs) such as methane, nitrous oxide, and carbon dioxide (Zahari et al., 2016).

Rice straw is made up of cellulose, hemicellulose, and lignin (Zahari et al., 2016). Due to abundant number of hemicelluloses in rice straw (20-40%), it can serve as the carbon source for the synthesis of xylanase (Gautam et al., 2018). Higher xylanase production can be induced with a substrate with higher content of xylan (Gautam et al., 2018) Besides that, Zahari et al. (2016) stated that rice straw is a suitable substrate that can be used to produce xylanase. Furthermore, research conducted by (Park et al., 2002) reported that *A. niger* KK2 supplemented with rice straw as carbon source produced a significance amount of xylanase which is a total of 5,071 IU/g of rice straw. Various researches also had been done to analyse

the synthesis of xylanase via solid state fermentation by using rice straw as one of the substrates in the process and the results from the study shown that promising amount of xylanases are being produced (Betini et al., 2009; Kanimozhi & Nagalakshmi, 2014; Kheng et al., 2004).

The study that had been done by Betini et al. (2009) described the production of xylanases from *Aspergillus niveus*, *A.niger* and *A.ochraceus* under SSF using several agro-industrial residues as substrate which include rice straw. Besides that, the SSF condition was not being optimised. The SSF process were done at incubation period of 30 °C at 70-80% for 96 hours. As for a study conducted by Kanimozhi & Nagalakshmi. (2014), the study done by them did not include initial moisture content of the substrate as the optimization parameters. It only included optimization of temperature, initial pH of medium, and effect of carbon and nitrogen source. Lastly, the study done by Kheng et al. (2004), focused on to optimize SSF parameters of *A.niger* USM A1 I by using palm kernel cake (PKC) as the substrate. As for rice straw substrate, the SSF conditions were not optimized.

3.0 MATERIALS AND METHODS

3.1 Preparation of rice straw

The substrate used to produce xylanase is rice straw, which was obtained from Kampung Batu 5, Jalan Sanglang, Kodiang, Kedah. The raw rice straw obtained were cut into small pieces before being cleansed thoroughly with tap water and distilled water to remove the dust and other contaminants. Then, the rice straws were dried in an oven at 60°C for 2 days. The dried rice straws were ground into smaller size by using electrical blender. Afterwards, the dried and ground rice straws were kept in the airtight containers for further use in SSF. Next, the rice straws were pre-treated with 1% (w/v) sodium hydroxide (NaOH) solution in the ratio of 1:2 (rice straw: solution) by soaking it in the solution for 2 hours. The pre-treated rice straws were then washed thoroughly with distilled water to remove any alkaline solution residues and dried in an oven at 60°C for 2 days.

3.2 Media preparation

Potato Dextrose Agar (PDA) was prepared for the sub-culturing of *A.niger* by dissolving 3.9 g of PDA powder with ultrapure water. Then, the solution was mixed well by using magnetic stirrer. Subsequently, the PDA solution was autoclaved at 121°C for 20 min. After the PDA solution had cooled down, ampicillin (50mg/ml) was added and the agar medium was poured into petri dish in laminar flow, followed by solidification of the agar at the room temperature

3.3 Preparation of reagent

The reagent used in this study such as 3,5-dinitrosalicylic acid (DNSA), 40% (w/v) of Rochelle's salt solution, 0.1 M sodium acetate buffer with pH 4.8, 1% xylan birchwood, and 1% xylose solution were prepared by using standard procedure.