CHARACTERISATION OF AMYLASE PRODUCED BY *Aspergillus Flavus* VIA SOLID-STATE FERMENTATION USING RICE HUSKS AS THE SUBSTRATE

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Bachelor of Science with Honours (Biotechnology Resource) 2014
Characterisation of amylase produced by *Aspergillus flavus* via solid-state fermentation using rice husks as the substrate

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A final report submitted in partial fulfilment of the Final Year Project 2

(STF 3015)

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Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2014
ACKNOWLEDGEMENT

Words are not enough to thank my supervisor. I would like to take this opportunity to acknowledge the guidance and encouragement of my supervisor, Miss Rosmawati Binti Saat, a lecturer in Department of Molecular Biology, Universiti Malaysia Sarawak. I have been inspired by her attention to detail and her energetic application to any problem. My heartiest thanks to my family, best friends, lab assistants and class mates for their concern and involvement that make me motivated along this study.
DECLARATION

I hereby declare that no portion of work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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<td>3,5- Dinitrosalicylic</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>°C</td>
<td>degree celcius</td>
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<td>%</td>
<td>percentages</td>
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<td>rpm</td>
<td>revolution per minute</td>
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<td>PDA</td>
<td>potato dextrose agar</td>
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<td>SSF</td>
<td>solid state fermentation</td>
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<tr>
<td>μm</td>
<td>micrometre</td>
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<tr>
<td>nm</td>
<td>nanometre</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>ml</td>
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ABSTRACT

The utilization of lignocellulosic agricultural waste namely rice husk has a high potential to act as a substrate under solid state fermentation by Aspergillus flavus. It supports the production of amylase at low cost and fewer labour works. This study examined specifically on the characterisation of Aspergillus flavus amylase activity using DNS assay method. Three parameters which are the temperature, period of incubation and pH were examined. The effect of incubation periods were done by varying the incubation of enzyme with 1% starch for 10, 20, 30, 40, 50 and 60 minutes. As for the temperature parameter, it was conducted at temperature of 20, 30, 37, 40, 50 and 60°C for 30 minutes. The study of pH influence on amylase activity was conducted with pH of 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0. The optimum amylase activity in DNS assay then were measured using spectrophotometric reading at absorbance wavelength of 540 nm and the analysis was done using glucose as a standard. The results showed that the Aspergillus flavus amylase activity were optimum at temperature of 37°C for 30 minutes and pH 5.0.

Keywords: Aspergillus flavus, amylase activity, solid state fermentation, rice husks.

ABSTRAK


Kata kunci: Aspergillus flavus, aktiviti amilase, penapaian keadaan pepejal, sekam padi.
1.0 INTRODUCTION

Rice husk is one of the most abundant agricultural wastes in Malaysia because the rice is the major food of its people (Noor Syuhadah & Rohasliney, 2011). Therefore, the use of rice husk as substrate in solid state fermentation is considered as an economic approach in the industrial sector. During fermentation, secreted amylase from Aspergillus flavus hydrolysed the component of cellulose in the rice husk to produce liquid glucose. The fungus, A. flavus was not fussy to be grown in the laboratory or bioreactor during solid-state fermentation. It needs less intensive care but it can reproduce asexually at the high rate Hedayati et al. (2007).

Amylase offered an alternative for the utilization of the agricultural by products to enzyme by bioconversion (Ritesh & Singhal, 2011). Therefore, amylase is the suitable enzyme to meet the industrial demands to hydrolyse cheaper carbon sources which is rice husk into the commercial valued end products, for example cooking sugar.

This study was conducted via solid-state fermentation method. This method was easier to be operated. It had a better outcomes compared to the conventional submerged fermentation, because it could produce more glucose (Barghav et al., 2008).

A. flavus amylase activities were determined using DNS method for enzyme assay (Mehrabadi & Bandani, 2009). DNS assay is the universal yet standard tool to measure the enzyme activity such as amylase (Bernfeld, 1955). There were three parameters, including the temperature, pH and incubation period that had been studied in order to characterise amylase produced by A. flavus under SSF with rice husk as the substrate.
This study was conducted with two objectives;

1. To characterize *Aspergillus flavus* amylase activity produced via solid-state fermentation using rice husks as substrate.

2. To determine the optimal temperature, pH and incubation period for the highest *Aspergillus flavus* amylase activity.
2.0 LITERATURE REVIEW

2.1 Amylase

Amylase is an enzyme consists of glucoamylase (EC 3.2.1.3) and α-amylase (EC 3.2.1.1) and it has a general function in hydrolysing complex carbohydrates such as starch into simple sugars, for example, maltose, maltotriose and glucose (Gautam et al., 2013). The simpler sugars are formed by the cleaving of 1, 4-α-D-glucosidic linkage between adjacent glucose units in the linear amylose chain by the aids of amylase. Thus, it has the high value in the industrial field due to this economic value. According to Gautam et al., (2013), amylase produced by *Aspergillus flavus* under solid-state fermentation is an amylolytic enzyme that is frequently used in food production industry.

2.2 Rice husk as substrate

Rice husks are the component of grain rice that serves the purpose of protection (Noor Syuhadah & Rohasliney, 2011). It is separated from the rice grain by the process of milling. The rice husk consists of lignocellulosic and opaline silica, therefore it is unable to digest by humans. In Malaysia, the annual output of rice is about 408,000 metric tonnes according to Malaysian Ministry of Agriculture (Noor Syuhadah & Rohasliney, 2011). Thus, the use of rice husks as the substrate in solid-state fermentation is the cost-efficient approach to get quality product. According to Dayanne et al. (2012), they also discovered that the enzymatic activities in rice husk were higher than other substrates such as wheat straw making the rice husk is the ideal substrate to use in fermentation process.
2.3 *Aspergillus flavus*

*Figure 1.0.* Macroscopic features of *A. flavus* in green colour on the agar plate. Adapted from http://www.pf.chiba-u.ac.jp/gallery/img/fungi/a/Aspergillus_flavus_colony.jpg.

*Aspergillus flavus* is the fungus with yellow to green or brown colour as shown in Figure 1 according to Hedayati *et. al.* (2007). It possessed conidiophores either biseriate or uniseriate, in various lengths, rough, pitted and spiny. Its conidia are globose to subglobose, varying from 3.5 to 4.5 μm in diameter and echinulated as referred in Figure 2.

*Figure 2.0.* Microscopic features of *A. flavus* showing its conidia. Adapted from http://www.mycology.adelaide.edu.au/images/flav2.gif
It spores were dispersed greatly by air movement or insect because of its airborne conidia. According to Vujanovic, et. al. (2001), the water activity ($a_w$) between 0.86 and 0.96 is where *A. flavus* grows better. It could grow optimally at the temperature of 37°C, (Hedayati et. al., 2007). *A. flavus* population are rapidly reproduced asexually because it was highly polymorphic in nature (Hedayati et. al., 2001). It has a higher activity against long chain substrates such as glycogen and starch because of its ability to secrete amylase for the hydrolysis of starch (Hedayati et. al., 2001).
2.4 Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is defined as a method of growing the microorganisms or fungi on the substrate without free flowing phase (Ghadi et al., 2011). It is an alternative to submerged fermentation to produce more quality products, for example enzymes, antibiotics, biofuels and biopesticides. It utilizes the agricultural wastes like rice husk for the substrate in the fermentation that acts as the carbon source for metabolism process. This method was cheaper than liquid state fermentation (LSF) because require less space, equipment and few man powers (Ghadi et al., 2011). SSF can even be done by using conical flasks. In addition, the risk of contamination can be reduced and the purification of products is easier as the crude enzymes were very concentrated. According to Barghav et al. (2008), most value added products are produced in higher amount by SSF compared to the submerged fermentation.
3.0 MATERIALS AND METHOD

3.1 Preparing reagents and substrates

3.1.1 3, 5-Dinitrosalicylic (DNS) reagent

150 ml glass bottle was wrapped with aluminium foil to prevent the reagent from oxidation due to sunlight ray. 100 ml of distilled water was poured into the 150 ml bottle. Then, 1 g of 3,5-dinitrosalicylic acid (DNS), 1g of sodium hydroxide (NaOH), 0.2g of phenol (C₆H₅OH₂) and 0.05g of sodium sulphite anhydrous (Na₂SO₃) were added into the bottle. The bottle was placed at room temperature and hidden from direct sunlight.

3.1.2 Acetate buffer (0.1M, pH 5.0)

250 ml glass bottle was filled with 200 ml of distilled water, then added with 2.5052g of sodium acetate (C₂H₃NaO₂). Then, the mixture was stirred on the hot plate. After that, the acetic acid was added drop by drop until the pH reaches 5.0 pH. The pH value was measured by pH meter. After the desired pH was achieved, the distilled was added up until the volume of the buffer reached 250 ml in the bottle. The buffer was placed inside the refrigerator at temperature of 4°C to avoid oxidation.

3.1.3 1% soluble starch

A glass bottle was filled with 80ml of distilled water, and then added with 1g of starch powder. The distilled water was top up until the volume reached 100ml. Then, the mixture was boiled until the mixture changed its appearance from cloudy to clear on the hot plate. After that, the solution was cooled and the bottle was wrapped with aluminium foil to avoid oxidation and direct sunlight.
3.1.4. 40% Potassium sodium tartrate solution (Rochelle’s salt)

40 g of potassium sodium tartrate was dissolved with 100 ml distilled water in the 100ml bottle.

3.1.5. 50 mg/ml ampicilin solution

Basic ratio for 1ml:
0.05 g of ampicilin powder dissolved in 1ml of ultrapure water.

Prepare for stock solution:
0.5 g of ampicilin powder dissolved in 10ml of ultrapure water.

Thus, according to ratio for PDA solution:
150μl of ampicilin solution for 150ml PDA solution.
3.2. Preparing of *Aspergillus flavus* culture

3.2.1. Preparing potato dextrose agar

Potato dextrose agar (PDA) powder (Merck's brand) was prepared in a conical flask by the addition of distilled water. This mixture was boiled and stirred for even dissolves using hot plate and stirrer. The conical flask was autoclaved. The media then was poured into petri dishes.

3.2.2. Culturing *Aspergillus flavus*

*Aspergillus flavus* were re-cultured on PDA (potato dextrose agar + 50 mg/ml ampicillin) plates. After that, the cultures were left at room temperature, 30°C for 5 to 6 days prior for inoculation.

3.3. SSF with rice husks as solid substrates

The rice husk was provided from the rice supplier in Kota Samarahan, Sarawak. It was ground into finer pieces. After that, the 5 g of rice husks was placed into 250 ml conical flask. Then, 10 ml of ultrapure water is used to achieve the initial moisture content of 70% (w/v).

The initial moisture was achieved by adding the sterilized distilled water. 3 plugs of *A.flavus* will be inoculated onto the rice husk as substrate in SSF. The incubation period was 6 days at the temperature of 30°C. The incubations were done in the incubator for ensuring the consistent and undistracted incubation process. The calculation to achieve 70% initial moisture content is included in the Appendix A.
3.4. Crude enzyme extraction

After the incubation was done, 20 ml of cold sodium acetate with the concentration of 0.1 M and pH 5.8 was added into the conical flask containing the culture and then shake at 120 rpm in room temperature. The earlier homogenized cultures were filtered through muslin cloth to separate the solution from other substrates and fungi. The filtered solutions were centrifuged at 6,000 rpm, at 4°C for 30 minutes. Then, the supernatant was collected. The supernatant was filtered through the filter paper for discarding any impurities. The filtration was done twice for the best result before the crude enzyme extracts were obtained. After that, the crude enzymes were further analysed.

3.5. Enzyme Assay of Crude Enzyme

Enzyme assay of the crude enzyme were carried out by DNS method following the procedures listed by Bernfeld (1955). In this method, the 0.5 ml of crude enzyme was reacted with 1 ml of 1% starch solution mixed with 0.5 ml of acetate buffer (0.1 M, pH 5.0) in the 15ml Falcon tube under standard conditions. Then, the mixture was incubated at 37°C for 30 minutes in the water bath. The reaction was then stopped by adding 2.0 ml of 3,5-Dinitrosalicylic (DNS) reagent inside the 15ml Falcon tube.

After that, the mixture was boiled in the water bath for 5 minutes and Rochelle's salt was added into the mixture after that to maintain the intensity of the mixture colour. The enzymatic activity was estimated using DNS assay whereby by estimating the amount of reducing sugar that is released and the glucose as a standard (Mehrabadi & Bandani, 2009).
3.6 Characterization of *Aspergillus flavus* amylase activity

Characterization of amylase activity was conducted by varying the parameters affecting the *A. flavus amylase activity* on DNS method, which includes temperature, pH and incubation time.

3.6.1 Effects of temperature on *A. flavus* amylase activity

The effect of temperature on the *A. flavus* amylase activity was determined by incubating the enzyme substrate reaction mixture in pH 5 at different temperature; 20, 30, 37, 40, 50 and 60°C for 30 minutes by using water bath.

3.6.2 Effects of pH value on *A. flavus* amylase activity

The effect of pH value on the *A. flavus* amylase activity was determined by mixing the enzyme substrate with different pH value of 0.1M acetate buffers; 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 at temperature of 37°C for 30 minutes.

3.6.3 Effects of incubation times on *A. flavus* amylase activity

The effect of incubation period on the *A. flavus* amylase activity was determined by incubating the reaction mixture at 37°C for 10, 20, 30, 40, 50 and 60 minutes.
3.7 Data analysis

The optical density (OD) readings were done by using spectrophotometer machine at the absorbance of 540 nm. The blank used for this experiment was 2.0 ml of acetate buffer. The blanks were used to calibrate the machine before the readings were taken. All the OD readings then were recorded. Then, the glucose concentration was calculated using glucose standard and further calculation on the total enzyme activity was done, using following formula:

\[
\text{Enzyme activity (U/ml)} = \frac{\text{Glucose concentration (mg/ml)} \times \text{Total volume of assay (ml)} \times \text{dilution factor (df)}}{\text{Volume of enzyme used (ml)} \times \text{The time of incubation (mins)}}
\]

The unit of total enzyme activity was measured in unit U per millilitre (U/ml). One example of the calculation using this formula is shown in Appendix B. The data were then tabulated in Tables 4.2, 4.3 and 4.4. Then, they are presented in the graphs as shown in Figures 4.2, 4.3 and 4.4.
4.0 RESULTS AND DISCUSSION

4.1 Culturing Aspergillus flavus

The culturing procedure of *A. flavus* was done in sterile and conducted under lamina floor hood to prevent any contamination such as foreign bacteria and fungus that might be grown on the culture. The ampicilin was added into the PDA solution to act as a treatment to prevent microbial infections (Liu *et. al.*, 2010). This ensured the pure culture of *A. flavus* was obtained.

4.2 Solid-state fermentation (SSF)

The fresh culture of *A. flavus* that had been grown within 6 days was the fungus at active stage (Hedayati *et. al.*, 2007). This type of culture was required to be inoculated inside the flask during SSF. This ensures the fungus to grow covering the whole surface of the rice husks inside the flasks. It helped to utilise the rice husks as its substrate during SSF for highest production of amylase.

The flask must be closed tightly to prevent the water vapour loss into the surrounding causing the rice husk to become slightly dry. This dry condition prevented the *A. flavus* from growing optimally because it needed humid condition for faster growth (Hedayati *et. al.*, 2007).
4.3 Glucose standard curve

The glucose standard curve was used as a tool to indicate the concentration of glucose present at different temperature, incubation periods and pH. According to Beer-Lambert’s Law, the resulting line that indicating the concentration of glucose versus absorbance should be straight or linear.

Table 4.1: Effect of different concentration of glucose on the absorbance of spectrophotometer

<table>
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<tr>
<th>Concentration of glucose</th>
<th>Absorbance (nm)</th>
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<tr>
<td>0.2</td>
<td>0.374</td>
</tr>
<tr>
<td>0.4</td>
<td>0.589</td>
</tr>
<tr>
<td>0.6</td>
<td>0.781</td>
</tr>
<tr>
<td>0.8</td>
<td>0.920</td>
</tr>
<tr>
<td>1.0</td>
<td>1.138</td>
</tr>
</tbody>
</table>

Based on following equation, the glucose concentrations in different parameters were calculated;

\[ y = 0.9295x + 0.2027, R^2 = 0.9955 \]

The glucose standard curve was presented in the Figure 4.1. The resulting line was appeared in the straight line following the Beer-Lambert’s Law. This result indicates that the glucose standard obtained was reliable and acceptable to be used for estimating the glucose concentration of each parameter tested for this study.
Glucose Standard Curve

$y = 0.9295x + 0.2027$

$R^2 = 0.9955$

Figure 4.1: The standard curve for glucose
4.4 Characterisation of *Aspergillus flavus* amylase activity

The characterization of *A. flavus* amylase activity was performed on several parameters such as incubation time, temperature of incubation and pH. The parameters were tested in DNS assay according to protocol introduced by Bernfeld (1955).

DNS actually stop the enzyme-substrate reaction between the 1% soluble starch and the amylase enzyme produced by *A. flavus*. Then, DNS react with reducing sugars, as the mixture was boiled up for approximately 5 minutes to develop the colour in the assay before going to optical density reading by spectrophotometer. 1ml of 40% Potassium sodium tartrate solution or Rochelle's salt was added to stabilize the colour intensity of the assay mixture by reducing the tendency of dissolved oxygen to increase the concentration of ion in the mixture solution.

NaOH present in the DNS reagent act as the agent for redox reaction between glucose produced by *A. flavus* amylase activity and DNS.