OPTIMISATION OF SSF PARAMETERS FOR CELLULASE PRODUCTION BY *Aspergillus spp.* VIA SOLID STATE FERMENTATION USING CORN HUSK AS THE SUBSTRATE

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Bachelor of Science with Honours Resource Biotechnology
2022

Optimisation of SSF Parameters for Cellulase Production by *Aspergillus spp.* Via Solid-State Fermentation Using Agro-Waste as the Substrate

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A project report submitted in partial fulfilment
of the Final Year Project 2
(STF 3015) course

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WS47 Resource Biotechnology Programme

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak 2022

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ACKNOWLEDGEMENT

First and foremost, I am appreciated that I could join the family of Molecular Biology lab 10. I would like to thank my supervisor, Dr Rosmawati binti Saat, who was willing to take me as her final year project student. She always guide and assist me during my research with patience; also provided motivation and moral support as she visited the lab. Besides, she also sent her helpful and approachable postgraduate student, Miss Alexandra Cheryl to guide us throughout the research. I would also love to express my gratitude to Miss Alex for her willingness to guide me whenever I need her and sacrificing her time to help me.

Next, I would like to thank my laboratory mates that conducting similar research as I did, with special thanks to Ong Liling, Steffi, and Nur Aidil Hakimy for sharing knowledges and helping me during the research. The other laboratory mates such as Amalin, Shamimi, Sharmina, and Rammiya for helping me during preparation stage and also provided moral support when I am stressed. Lastly, special thanks to my parents who always be there for me, providing financial support and moral support with their loves and encouragement.

Optimisation of SSF Parameters for Cellulase Production by *Aspergillus spp.* Via Solid-State Fermentation Using Corn Husk as the Substrate

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ABSTRACT

Malaysia produced few thousand metric tons of agro waste annually; the improper waste disposal causes environmental pollution. Thus, bioprocess industry should take agro waste as the substrate for bioprocess to produce enzyme such as cellulase. Cellulase is known as an economical important enzyme in various industries. As the demand for cellulase is increasing due to the development of biofuel industry, the optimisation of solid state fermentation parameters to produce high quality cellulase with lower production cost and higher efficiency need to be studied. In this study, *Aspergillus niger* was used to perform SSF with corn husk as substrate to produce cellulase. The cellulase activity at different optimised SSF parameters such as initial moisture content, incubation period and incubation temperature were assayed by filter paper activity (FPA) assay. The FPA was used to test the cellulase activity and the amount of glucose released. The optimum temperature for the highest cellulase enzyme activity of *A. niger* grown on corn husk was at 30°C while optimum initial moisture content was 65% (v/w) and 6 days was the optimum incubation period. The highest enzyme activity was 47.2222 ± 5.6 U/ml. The results suggest the potential of corn husk to be utilised for industrial purpose to produce cellulase using *A. niger*.

Keywords: agro waste, *Aspergillus niger*, cellulase, corn husk, solid state fermentation

ABSTRAK

Setiap tahun, beberapa ribu tan metrik sisa agro dihasilkan di Malaysia dan pengurusan pembuangan sisa yang tidak sesuai telah menyebabkan pencemaran alam sekitar. Oleh itu, sisa-sisa agrokultur harus digunakan dalam industri bioproses untuk menghasilkan enzim seperti selulase supaya memanfaaatkan alam sekitar. Selulase merupakan enzim yang mempunyai kepentingan ekonomi dalam pelbagai industri seperti industri biofuel. Maka, optimasi faktor-faktor fermentasi berkeadaan pepejal harus dikajikan demi menghasilkan selulase yang berkualiti dengan lebih efisien supaya dapat mengurangkan kos pengeluaran. Dalam kajian ini, selulase dihasilkan oleh Aspergillus niger *dengan menggunakan sekam jagung sebagai substrat melalui fermentasi berkeadaan pepejal. Esei kertas penapis digunakan untuk mengenalpasti aktiviti enzim dalam factor-faktor fermentasi berkeadaan pepejal (kandungan pelembapan awal, tempoh pengeraman dan suhu) yang telah dioptimasikan. Esei kertas penapis digunakan untuk menkaji aktiviti enzim dan jumlah glukosa yang dibebaskan. Melalui fermentasi berkeadaan pepejal pada suhu 30°C, kandungan pelembapan awal 65% (v/w) and tempoh pengeraman selama 6 hari, aktiviti selulase maksimum* Aspergillus niger *yang bertumbuh di sekam jagung (47.2222 ± 5.6 U/ml) telah diperolehi. Potensi penghasilan selulase menggunakan sekam jagung dalam industri telah dibuktikan melalui kajian ini.*

Kata kunci: sisa agro, Aspergillus niger*, selulase, sekam jagung, fermentasi berkeadaan pepejal*

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List of Abbreviations

A. niger	*Aspergillus niger*
°C	Degree celcius
DNS	Dinitrosalicylic acid
EMC	Existing moisture content
FAO	Food and Agricultural Organization of United Nation
FPA	Filter paper assay
FPase	Filter paper hydrolase
IUPAC	International Union of Pure and Applied Chemistry
mg/ml	Microgram per millilitre
ml	Milliltre
nm	Nanometre
OFAT	One factor at a time
PDA	Potato Dextrose Agar
rpm	Revolution per minute
SMF	Submerged-liquid fermentation
NaOH	Sodium hydroxide
SSF	Solid state fermentation
S.D.	Standard deviation
U/ml	Unit per millilitre
w/v	Weight over volume
a_w	Water activity

1.0 INTRODUCTION

1.1 Background

The usage of microbes dated back to 6000 BC where Sumerians and Babylonians utilised yeast in the fermentation of alcoholic beverages (Singh, 2016). Eduard Buchner discovered that zymase is the enzyme responsible for catalysing the reaction of fermentation (Alba-Lois & Segal-Kischinevzky, 2010). Ejaz et al. (2021) mentioned that all living organisms could produce enzymes. These enzymes are widely used in industry when green chemistry is employed to replace hazardous chemical usage.

Cellulase is one of the important microbial hydrolytic enzymes in industry, especially cellulases originating from three major genera of filamentous fungi such as *Penicillium, Tricoderma* and *Aspergillus* (Singh, 2021). The three major categories of cellulase are β- glucosidase, exoglucanase and endoglucanase are responsible to cleave the β-1,4-glucosidic bond of the cellulose polymer (Zhang & Zhang, 2013) to hydrolyse cellulose to glucose. Cellulase has been utilised in many industry sectors such as biofuel, brewing, agriculture, food processing, textile, pulp and paper and more. The Global Cellulase (CAS 9012-54-8) Market Research Report done by QY research (2018) forecasted that the market size of cellulase will increase from 1500 million USD (2018) to 2300 million USD in 2025.

In recent decades, the studies and application of producing biofuel from lignocellulosic biomass have become a new trend. The expansion of the biofuel industry leads to an increase in demands of cellulase as it is the second most crucial enzyme in the production of biofuels (Siqueira et al., 2020). Lignocellulosic biomass consists of a complex lignocellulose structure which is made up of cellulose, lignin and hemicellulose. The crystalline structure of cellulose leads to low solubility in water;

thus, the biomass is difficult to hydrolyse. An example of lignocellulosic biomass is agro waste such as rice husks, corn husk, and sugarcane bagasse, consisting of high cellulose, hemicellulose and lignin composition (Isikgor & Becer, 2015). Thus, cellulase is used in the pretreatment of lignocellulosic biomass to hydrolyse the polymer to monomer (glucose), which can be used in fermentation (Siqueira et al., 2020).

Solid state fermentation (SSF) is an eco-friendly method for the production of cellulase as it requires low expenses and is capable of producing a higher yield by optimising the use of substrate compared to submerged-liquid fermentation (SMF) (Martăuet al., 2021). SSF refers to the growth of microorganisms on a solid substrate with low water content or water-free (Webb & Manan, 2017). The substrate used for SSF is generally agro waste which is cheap and abundant, thus, the problem of disposal of agricultural waste to the environment can be alleviated. However, the choice of microorganisms used in SSF depends on the desired final product, type of substrate used and the growth requirements (Martăuet al., 2021).

The filamentous fungi is the best microorganism to be cultured with SSF due to their physiological, enzymological, and biochemical properties. In addition, the nature of fungi to colonise and utilise agro waste makes them fit the requirement of SSF due to the capability to grow their hypha on the surface and deep into the solid substrate. One of the most used filamentous fungi is *Aspergillus niger*. However, the growth rate and yield of the final product can be influenced by various factors or parameters (Webb & Manan, 2017).

Webb and Manan (2017) mentioned that physio-chemicals, biological, and mechanical factors are the three major categories of SSF parameters. The physio-chemical factors are the most studied parameters, such as initial moisture content,

incubation period, temperature, pH, and more. A study done by Jimat et al. (2018) showed that the SSF parameters affected the SSF process. Hence, the conditions of SSF for the production of cellulase need to be studied as to increase its enzyme activity. In this study, SSF parameters optimisation is studied using corn husk as substrate as it is easy to access in our daily life, and *A. niger* is used for cellulase production via SSF. By using the filter paper assay (FPA) the cellulase activity can be evaluated.

1.2 Objectives

1. To investigate the optimum conditions for SSF by using alkali pretreated corn husk in order to obtain maximum cellulase enzyme activity from *A. niger*.
2. To optimise the selected SSF parameters (incubation temperature, initial moisture content and incubation period) for the cellulase production by one factor at a time (OFAT).

2.0 LITERATURE REVIEW

2.1 Cellulase

Cellulase is a group of enzymes found in the natural environment. Microorganisms such as bacteria and fungi are the main producer of cellulase for industrial purposes (Ray &Behera, 2017). According to Zhang and Zhang (2013), β-glucosidase, exoglucanase and endoglucanase are major cellulases responsible for cleaving the β-1,4-glucosidic bond of the cellulose polymer. These three cellulases have different modes of action during the hydrolysis of cellulose. The endoglucanases are capable to cleave the cellulose chain randomly at amorphous regions producing oligomers, exoglucanases break linkages of oligomers to produce cellobiose at the reducing and non-reducing ends, B-glucosidase hydrolyse the nonreducing ends of cellobiose and release glucose (Figure 1) (Siqueira et al., 2020). Fungi are often used in cellulase production as fungi produce higher yields than other microorganisms; the most studied fungi are *Aspergillus* and *Trichoderma*.

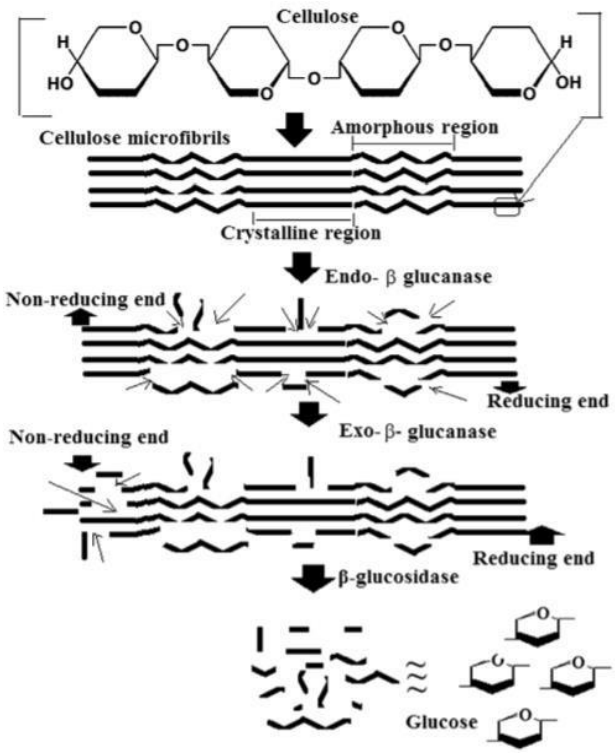

Figure 1. The sequential stages in the hydrolysis of cellulose (Brahmachari, 2017).

The Solid State Fermentation technology is often used to produce fermented foods such as soy sauce, tapai and sake in the Orient and Asian region; this technology has been practised for centuries and is still remained until now (Webb & Manan, 2017). In recent decades, SSF has beenimproved and used in large-scale production for industrial purposes but the optimisation of SSF parameters to increase yield is a long-term study. According to Wedd and Manan (2017), the advantages of SSF can be categorized into four categories based on different criteria which are biological, processing, environmental and economic advantages (Table 1).

Table 1. The advantages of SSF (Martău et al., 2021).

Type of Advantages	Details
Biological	• Contamination is less likely to occur due to low water content. • Higher yields and productivities. • Products with higher stability.
Processing	• Simple process with less effluent release. • Simpler downstream process. • Less solvent is needed to extract products. • The generation of hazardous waste is minimized.
Environmental	• Minimized the production of liquid waste. • Uses natural materials as the substrate. • Alleviate the waste problem.
Economic	• Lower capital and operating costs.

As mentioned by Martău et al. (2021), SSF offers more advantages over the SMF in terms of technology and economics; more research needs to be done in future for improvement (Webb & Manan, 2017). There are a few differences between SSF

and SMF (Table 2) that reveals the advantages of SSF.

Table 2. Characteristics differences between SSF and SMF (Webb & Manan, 2017)

SSF	SMF
There is no free water, and the water content of substrate is in the range 12-70%	Water is the main component of the culture
Microorganisms absorb nutrients from the wet solid substrates; a nutrient concentration gradient exists	Microorganisms absorb nutrients from the liquid culture; there is no nutrient concentration gradient
The culture system consists of three phases (gas, liquid and solid) and gas is the continuous phase	The culture system mainly consists of liquid; the liquid is the continuous phase
Inoculation size is large, more than 10%	Inoculation size is small, less than 10%
The required oxygen is from the gas phase; the process needs low energy consumption	The required oxygen is from dissolved oxygen; there is a larger amount of dissolved oxygen
Microorganisms attach and penetrate into the solid substrate	Microorganisms uniformly distribute in the culture system
At the end of fermentation, the medium is a wet state substrate, and the concentrations of products are high	At the end of the fermentation, the medium is liquid and the concentrations of products are low
High production rate and high product yield	Low production rate and low product yield
Mixing is difficult or impossible, some microorganisms are sensitive to mixing or agitation and the growth of microorganisms is restricted by nutrient diffusion	Mixing is easy, and the growth of microorganisms is not restricted by nutrient diffusion
Removal of metabolic heat is difficult	Temperature control is easy
Heterogeneity	Homogeneity
The fermentation parameters are hard to detect and control on-line	The fermentation parameters can be detected and controlled on-line
Extraction process is simple and controllable; little waste water	Extraction process is usually complex; there is a large amount of waste water
Low water activity	High water activity
Simple fermentation bioreactor	High-tech design fermentation bioreactor
Natural enrichment or artificial breeding systems	Pure strains
Energy consumption and equipment investment are high	Energy consumption and equipment investment are low
Low raw material cost	High raw material cost

2.1.1 SSF Parameters

The productivity of SSF products can be influenced by numerous factors. The parameters mentioned in this study are initial moisture content, incubation temperature

and incubation period. The water requirements of microorganisms for microbial activity can be expressed quantitatively in the form of water activity (a_w) of the environment or substrate. The a_w value represents the level of free water in the substrate, it can be used to determine if the microorganism chosen is suitable for SSF. The microorganisms with lower a_w value such as filamentous fungi (0.6 - 0.7) are adaptable in SSF (Webb & Manan, 2017). Microorganisms such as bacteria need high a_w which is around 0.9 while yeasts grow at the value of 0.8. The heat transfer capacity is limited within the solid substrate, which has poor thermal conductivity. Thus, at low moisture content and leads to different temperatures all over the medium. The most efficient process for temperature control is water evaporation. Aeration during aerobic SSF also can increase the rate of heat and moisture transfer between the solid and the gas phase, together with the supply of oxygen (Webb & Manan, 2017). Webb and Manan (2017) also mentioned that the incubation period in SSF depends on the type of microorganisms chosen.

2.2Agro waste

Agro waste also called agricultural waste is the residues of the raw agricultural products during the growing or processing of products such as crops, poultry, vegetables, meat and more. Agro waste normally consists of crop waste, animal waste, food processingwaste, and hazardous and toxic agriculture waste (Obi et al., 2016). Corn husk is included in the category of crop waste. According to Pessanha da Penha et al. (2012), agro waste is the best substrate for SSF as it is cost-effective; the use of agro waste for biotechnology purposes could reduce environmental pollution due to improper disposal.

2.2.1 Corn Husk

Corn husks are the outer layer of green leaves that wrapped around the corn cob.

According to Ibrahim et al. (2019), corn husk provided greatest level of cellulose content compared to other parts of the corn plant. Sharma et al. (2018) states that the chemical composition of corn husk consists of cellulose (35.67 %), hemicellulose (25.46 %) and lignin (21.05 %). The research done by Ibrahim et al. (2019) also revealed that corn husk contained cellulose (45.7%), hemicellulose (35.8 %) and lignin (4.03 %). It is an ideal source of the substrate in SSF due to its high lignocellulosic composition. It is important to utilize agro waste using biotechnology as the production of agricultural waste in Malaysia is predicted to reach 0.210 (kg/cap/day) by 2025 and currently most of these agro wastes are disposed into landfill (Siddiqui & Naidu, 2019).

2.3 *Aspergillus niger*

A. niger is an asexual filamentous ascomycete fungus under the genus of *Aspergillus*.The reproductive part of *A.* niger, the conidial heads (Figure 2) produce dark or dark brown spores from which none of the *Aspergillus spp.* present this characteristic. The vegetative part of *A. niger*, hyphae absorb nutrients from dead or decaying and grow on it. It *A. niger* grows and spreads very fast at 30–35°C, the desirable a_w value is 0.88 and able to grow within 1.4 to 9.8 pH range (Schuster et al., 2002). In 1919, the *A. niger* strain was first applied to produce citric acid by fermentation as it produced minimal of by-products and cheap. Later, it was used to produce various enzymes using surface culture. According to Schuster et al. (2002), Food and Agriculture Organization (FAO) approved *A. niger* to be used in food industry which it is a safe organism. However, rare cases might happen where the *A. niger* may infect

human who are immunocompromised.

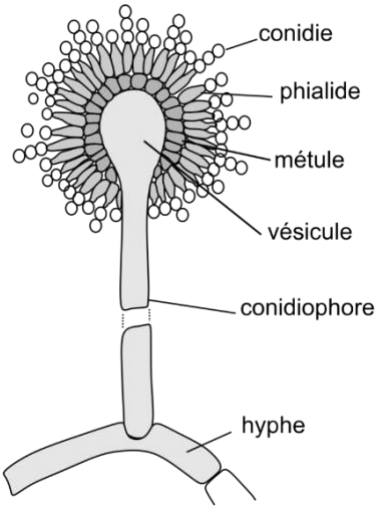

Figure 2. Morphology of *A.niger*. (Mokobi, 2021).

2.4 Filter Paper Activity (FPA)

FPA assay is an International Union of Pure and Applied Chemistry (IUPAC) established method that is commonly used to evaluate the cellulase activity by using the Whatman No.1 filter paper as the substrate (Yu et al., 2016). The important point of the assayis that a fixed amount of glucose (2mg) is required for the assay, thus, the criteria of filter paper is fixed to be 50 mg (1 x 6 cm). Besides, a series of cellulase dilution solutions is required to achieve a fixed degree of hydrolysis. The main idea of the IUPAC method is that cellulase must be diluted until the amount of product plotted against cellulase concentrationis reasonably linear (Yu et al., 2016).

3.0 MATERIALS AND METHODS

3.1 Pre-treatment of substrate

Corn husk was selected as the substrate and was collected from a farm located at kampung Asajaya, Sarawak. The corn husk collected were filtered and rinsed with tap water, followed by distilled water twice to remove dirt, unusable part, and possible contaminants. Then, the substrate was dried in the drying oven at 60°C until it became papery texture. The dried corn husk was cut and grounded into smaller pieces using blender. The ground substrate was kept in airtight container for further use. Next, the substrate was pre-treated with 4% (w/v) sodium hydroxide (NaOH) solution by mixing the substrate and NaOH at 1:2 ratio in the 250ml Erlenmeyer flask at room temperature for 2 hours. Distilled water was added to the flask after 2 hours to stop the reaction and later rinsed with distilled water to remove the excess alkaline solution. The pre-treated substrate was dried at 60°C in drying oven.

3.2 Media preparation

Potato Dextrose Agar (PDA) was used for sub-culturing *A. niger*. Thus, 8.58 g of PDA powder was dissolved in 220ml of ultrapure water and magnetic stirrer was used to mix the PDA solution. Then, the PDA solution was autoclaved at 121°C for 20 minutes. The PDA solution was let cooled and 220µl ampicillin (50mg/ml) was added prior to the pouring of PDA agar medium into petri dish. Pouring of PDA agar medium was done in laminar flow and was let to solidify at room temperature.

3.3 Fungal cultivation

Aspergillus niger was obtained from the fungal collection of Molecular Biology Laboratory, Faculty of Resource Science and Technology, University Malaysia Sarawak (UNIMAS). The *A. niger* strain was sub-cultured obtained by growing it on PDA solid medium and incubated at room temperature until fully grown and matured (Figure 3.1). The culture was kept at 4°C before use.

Figure 3.1. Matured *A. niger*.

3.4 Solid State Fermentation (SSF)

SSF were carried out by adding 5g of substrate into Erlenmeyer flasks. The initial moisture content was adjusted to 70% (v/w) then incubated at temperature of 30°C for 6 days. The amount of distilled water added to reach 70% (v/w) initial moisture content was calculated as shown in **Appendix B**. The substrate was inoculated with 3 plugs of 6 days sub-cultured *A. niger* using inoculum loop (Figure 3.2). The experiment was done in triplicate, and the results were presented as the mean for every three replicates.

Figure 3.2. Corn husk inoculated with 3 plugs of sub-cultured *A. niger*.

3.5 Enzyme extraction

Extraction of enzyme were done by adding 20 ml of 0.1 M cold sodium acetate buffer (pH 5.5) into the flasks. Flasks were agitated on the rotary shaker for 30 minutes, 120 rpm at room temperature. The mixture was filtered with muslin cloth into the falcon tube on ice and sent to centrifuge at 10000 rpm for 15 minutes at 4°C. Later, the supernatant was collected and filtered twice using Whatman filter paper to obtain crude enzyme to be used directly for enzyme assay

or stored at 4°C before used.

3.6 Enzyme Assay

The filter paper assay (FPA) method is adapted from Mrudula and Murugammal (2011), test tube contained Whatman No. 1 filter paper strips, crude enzyme (1 ml) and 0.1 M citrate buffer with a pH of 5.5 (1 ml) was incubated at 50°C for 30 minutes using a water bath. The Whatman No. 1 filter paper strips (50 mg; 1x6 cm) was used to assay the cellulase activities. The dinitrosalicylic acid (DNS) reagent (2 ml) was added then being boiled for 15 minutes, immediately added with 1 ml of 40% (w/v) Rochelle salt to stabilize the colour. The absorbance value was measured at 540 nm by a spectrophotometer with 1.5 ml of sample in the cuvette. One mole of reducing sugar produced from filter paper per ml/min was one filter paper unit (PFU). Glucose standard curve (**Appendix C**) was prepared to determine the concentration of reducing sugar in the sample. The formula to calculate the total enzyme activity (U/ml) as shown below:

$$\frac{\text{Reducing sugar released (mg)} \times \text{Total assay volume (ml)} \times \text{Dilution factor}}{\text{Volume of enzyme used (ml)} \times \text{Volume of sample in cuvette (ml)} \times \text{Incubation time (min)}}$$

3.7 Optimization of SSF parameters

3.7.1 Effect of initial moisture content

The optimum cellulase activities at a different initial moisture content of 60 %, 65%, 70%, and 75% (v/w) were assayed after incubated for six days at 30°C. The moisture content level was adjusted by adding distilled water, the calculation for distilled water added shown in **Appendix B**. The process of fermentation, enzyme extraction and enzyme assay were performed according to sections 3.4, 3.5 and 3.6.

3.7.2 Effect of incubation period

The optimum cellulase activities at different incubation periods ranging from 0 to 6 days were assayed under the condition where the initial moisture content was 70% (w/w) and incubated at 30°C. The moisture content level was adjusted by adding distilled water, the calculation for distilled water added shown in **Appendix B**. The process of fermentation, enzyme extraction

and enzyme assay were performed according to sections 3.4, 3.5 and 3.6.

3.7.3 Effect of incubation temperature

The optimum cellulase activities at different incubation temperatures of 25°C, 30°C, 35°C, 40°C, 50°C, and 60°C were evaluated after incubated for six days at the initial moisture content of 70% (w/w). The moisture content level was adjusted by adding distilled water, the calculation for distilled water added shown in **Appendix B**. The process of fermentation, enzyme extraction and enzyme assay were performed according to sections 3.4, 3.5 and 3.6.