# UTILIZATION OF WATERMELON PEELS AS A SUBSTRATE FOR ALPHA AMYLASE PRODUCTION BY BACILLUS SUBTILIS IN SOLID STATE FERMENTATION

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# UTILIZATION OF WATERMELON PEELS AS A SUBSTRATE FOR ALPHA AMYLASE PRODUCTION BY BACILLUS SUBTILIS IN SOLID STATE FERMENTATION

**by**

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction

Enzymes are unique biocatalysts that speed up a chemical reaction and have highly specific proteinaceous substance as an active site. Alpha amylase E.C.3.2.1.1, endo-1, 4-α-D-glucan glucohydrolase is one of the most widely used enzymes in the industry. Figure 1.1 shows the structure of alpha amylase. These enzymes are endo-acting enzymes which randomly hydrolyse α-1-4-glycosidic bonds between adjacent glucose units in a starch polymer leading to the formation of linear and branched oligosaccharides such as glucose, maltose and maltoriose (Swargiari and Baruah, 2012). Alpha amylase originally produced in bacteria, fungi, plants and animals. However, production by microbial provides efficient production strategies over the other sources such as commercial bulk production capacity and easy to manipulate to obtain enzyme of desired characteristics (Souza, 2010). These enzymes contribute about 30% of the world's enzyme production (Van Der Maarel et al., 2002). This shows the important role of alpha amylase in biotechnology enzyme usage. This enzyme has a great significance in biotechnology with applications ranging from food, textile, alcohol, detergent and paper industries (Bruins et al., 2001). In addition, present day alpha amylase has been extensively applied in clinical, medical and analytical chemistry as well due to advances in biotechnology (Souza, 2010).

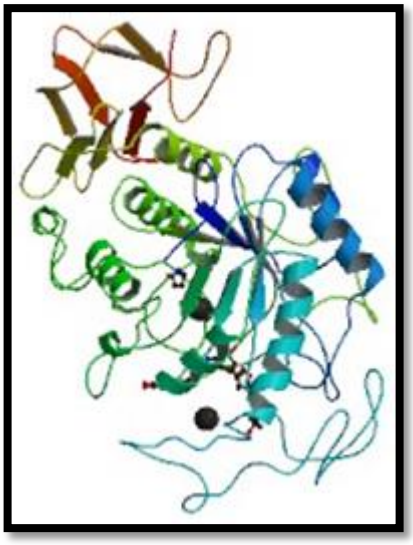

Figure 1.1: Structure of alpha amylase (Singh et al., 2011)

Watermelon or its scientific name *Citrullus lanatus* is grown widely in tropical and subtropical countries. The fleshy pulps of these fruits are consumed leaving behind the seeds and the peels. It is generated from the restaurants, small scale fruit juice procedures, fruit sellers and these wastes are not much being reused. This results the great amount of agro-industrial wastes which is peels generated during the processing and their disposal rather causes several environmental problems. Therefore, there is the need for a research in the development of the possible industrial potential of fruit wastes. Most of the studies on watermelon fruits have focused on the anti-nutritional (Johnson et al., 2012) and phytochemical and anti-oxidant properties of the fruit juices (Oseni and Okoye, 2013). In addition, in Malaysia watermelon peels have been analysed and reutilized as potential raw materials for production of jam (Souad et al., 2012). Application of agro-industrial residues in bioprocesses provides alternate substrates besides solving pollution problems. Table 1.1 shows list of various agro-industrial wastes used to produce different products by microorganisms. The watermelon peels

(WP) can be used as substrate specifically carbon source in the fermentation process on production alpha-amylase. It is utilized as raw material as they provide a good substrate for the growth of *Bacillus subtilis*.

Table 1.1: List of various agro-industrial wastes used to produce different products by microorganisms (Singhania et al., 2008)

| Type of agro-industrial wastes | Microorganisms | Products |
|---|---|---|
| Pineapple, mixed fruit and mausami waste | *Aspergillus niger* | Citric acid |
| Apple pomace | *A. niger* | Pectin methylesterase |
| Apple pomace | *A. niger* | Citric acid |
| Apple pomace | *Thamnidium elegans* | ϒ-linolenic acid |
| Orange peels | *Rhizopus oryzae* | Pectin lyase |
| Orange peels | *Curvularia inaequalis* | Pectin lyase |
| Cucumber fruits | *Pythium splendens* | Pectin lyase |
| Orange pulp pellets | *Tubercularia vulgaris* | Pectinase |
| Potato pulp | *R. oryzae* | Lactic acid and ethanol |
| Apple and strawberry pomace | *Lentinus edodes* | Polygalactouranase |
| Banana stalk waste | *B. subtilis* | Cellulase |
| Grape pomace | *A. awamori* | Hydrolytic enzyme |
| Kiwi fruit waste | *Trametes hirsute* | Laccase |

Solid state fermentation (SSF) is one of techniques for production of alpha-amylase. It uses solid substrate waste that containing nutrient-rich as substrate. The use of fruit waste as a substrate makes SSF as an attractive alternative method. It is more economically available agricultural and industrial by-products such as bran, bagasse, fruit seed and fruit peel, as they are considered to be good substrates for SSF to produce enzymes. Substrates are used very slowly and consistently during prolonged fermentation periods. SSF technique is preferred because of numerous advantages such as simple fermentation equipment, low cost substrates and suitable for fungi and bacterial species that required less moisture content. Furthermore, it produce higher yields in a shorter time period, better oxygen circulation and less effluent generation (Swargiari and Baruah, 2012).

*Bacillus* species are industrially important microorganisms because of their rapid growth rate that lead to short fermentation cycles and generally handling safety (Pandey et al., 2000). Microbial sources are being exploited for alpha amylase production in SSF but only a few species of *Bacillus* and their improved strains have the ability to produce alpha amylase in commercial scale such as *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens* and *B. steatothermophilus* (Souza, 2010). Thus, this microbial species is reflected to be the most important sources for enzyme production, alpha amylase.

## 1.2 Problem Statement

The fruit wastes are a major challenge to the food industry where need to consider the waste dumping. Normally they are simply burnt or dumped to rot, which

poses a threat to human health and the environment. Agro-industrial wastes are mainly composed of complex polysaccharides that might serve as nutrients for microbial growth Thus, these wastes in bioprocess perform really to be compliant, as their effective utilization for the production of organic acid, especially citric acid, and other products such as enzymes (Singhania et al., 2008). From the watermelon itself, the peel and seeds are accumulated as wastes in the environment, posing serious environmental problem. Hence, this project has been proposed to utilize the WP as the substrate for alpha-amylase production. It contains 56.02% carbohydrates content (Al-Sayed and Ahmed, 2013) which can act as carbon source to enhance production of alpha amylase.

Fermentation period primarily depends on the characteristics of the culture organisms and enzyme production pattern. Elongated fermentation is not advantageous since it can lead to loss of moisture (Swargiari and Baruah, 2012). Moreover, pH is an important factor which also affects the growth and enzyme production during SSF. The growth and production of alpha amylases occur at nearly neutral pH for bacterial species. The pH range of the enzyme stability is within pH 5-7 (Saxena and Singh, 2011). It is reported by Unakal et al. (2012) substrate concentration in fermenting media raises the production of enzyme up to optimum level. Beyond the optimum level of substrate would not increase the enzyme yield and could not affect the growth of organisms.

Most reports reveal that the production of alpha amylase over SSF fermentation media consisting of various agro-industrial wastes supplemented some mineral salts that are necessary for the boosted secretion of the enzyme. The main goal of this study is to determine the potential of WP as a substrate for production of alpha amylase by *B.*

*subtilis* under SSF. Moreover, the effect of enzyme production parameters; incubation period, pH and substrate concentration were studied.

### 1.3 Research Objectives

The main objectives of this study are:

i. To preliminary study and compare alpha amylase production between watermelon peels and jackfruit seeds.

ii. To optimize process parameters for alpha amylase production under solid state fermentation by *B. subtilis* using watermelon peels as a substrate.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction on alpha amylase

Amylases are most broadly used enzyme in industry. It hydrolyzes starch molecule to produce glucose and maltose. Alpha-amylase (E.C.3.2.1.1, endo-1, 4-α-D-glucan glucohydrolase) randomly hydrolyses α-1.4-glysidic bonds between adjacent glucose units in a starch polymer leading to the formation of higher oligosaccharides (Swargiari and Baruah, 2012). Figure 2.1 shows the enzymatic hydrolysis of starch to glucose. Thermostable alpha amylases are generally preferred as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving (Pandey et al., 2000). Amylases can be derived from various sources such as plants, animals and microorganisms. For an example, amylases from plant and microbial sources have been employed as food additives whereas fungal amylases have been widely used for the preparation of oriental foods (Sivaramakrishnan et al., 2006). Strains of *Aspergillus sp.* and *Bacillus sp.* are employed for commercial applications. Majority of the bacterial amylases are obtained industrially because of their short growth period (Pavithra et al., 2014). Therefore, the production of alpha amylase rises at the optimized conditions.

## 2.2 Microorganisms capable to produce alpha-amylase

Microbial sources, especially thermophilic bacteria and mesophilic molds provide industrial need of alpha amylases (Souza, 2010). *Bacillus* species are reported to

be the most important sources of alpha amylase and have been used for enzyme production using solid state fermentation (SSF) (Saxena and Singh, 2011). Table 2.1 shows lists of alpha amylase production by *Bacillus* species using different substrates. *Bacillus subtilis* was mostly studied bacterial strain in SSF for production of alpha amylase due to this strain produced high levels of thermostable alpha amylase with characteristics suitable for application in starch processing and food industries (Asgher et al., 2007). Thermostable enzyme was important as enzymatic liquefaction and saccharification of starch were performed at high temperature (Sundarram and Murthy, 2014). Ashok Pandey stated that *Aeromonas caviae* is also one of the bacteria species that producing alpha amylases under SSF (2003). There have been increase studies of SSF techniques to produce industrially important enzymes including alpha amylase (Lonsane and Ramesh, 1990). SSF imitates the natural microbiological process such as composting and its controlled utilization could survive at low moisture content (Sivaramakrishnan et al., 2006). In the same way, fungal species along with some yeast have shown to produce alpha amylase such as *Aspergillus* and *Penicillium* (Souza, 2010).

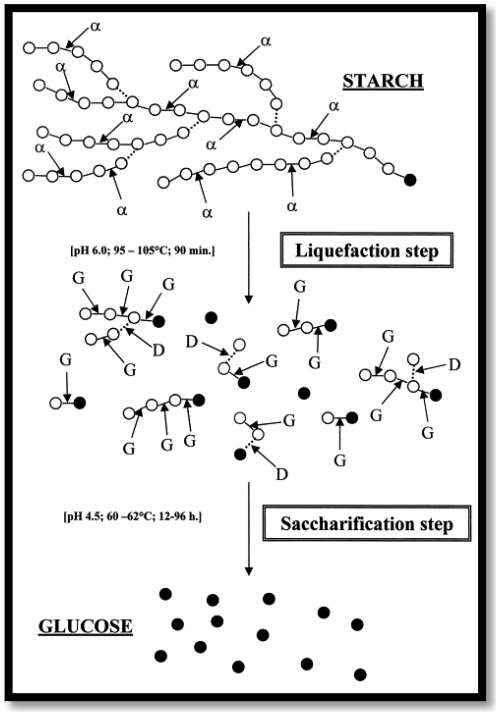


Figure 2.1: Industrial enzymatic hydrolysis of starch into glucose and pattern of action of amylolytic enzymes (Lévêque et al., 2000)

Table 2.1: Lists of alpha amylase production by *Bacillus* species using different substrates

| Subsrates | *Bacillus* species | References |
|---|---|---|
| Sunflower meal | *B. licheniformis* | Ikram ul et al. (2003) |
| Rice flake | *B. cereus MTCC 1305* | Anto et al. (2006) |
| Maize bran | *B. coagulans* | Babu and Satyanarayana (1995) |
| Rice husk | *B. subtilis* | Baysal et al. (2003) |
| Rice bran | *Bacillus sp* PS-7 | Sodhi et al. (2005) |

## 2.3 Agro-industrial waste products as substrate

There has been an increasing efforts to utilize agro-industrial wastes in biotechnological innovations mainly fermentation technology particularly those originating from tropical regions (Singhania et al., 2008). These developments open the opportunities for industrial economic utilization. Accordance to that, low-cost medium is required for the production of alpha amylases to meet the industries demand. Nutrient broth, soluble starch and other synthetic media are very expensive and should be replaced with cheaper agricultural by-products or waste for the reduction of the SSF medium cost. Application of agro-industrial waste in bioprocess is an ideal selection for enzyme production in SSF due to cost and availability of the substrate. This development was also capable to reducing its environmental impact. Table 2.2 shows alpha amylase production by different strains in SSF with different substrates. It shows that enzyme activity varies with the type of agro-industrial wastes used and the microbial culture because of different nutrient contents in the wastes as well as different capacity of microorganism to metabolite the supplied nutrients (Prakasham et al., 2006). Thus, it is important the agro-industrial wastes as substrates have good criteria such as its availability and nutrient content (carbon source) to suit the specific strain for optimize and maximize the enzyme production. Watermelon peels (*Citrullus lanatus*) and Jackfruit seeds (*Artocarpus heterophyllus*) are agro-industrial wastes generated by most food industries in Southeast Asia and particularly in Malaysia (Souad et al., 2012). Considering the easy availability of these wastes in our country, it seems that it is an opportunity for scientific study to fully exploit their benefits. On the other hand, this will reduce their waste dumping problem. WP and JS are currently less utilized as a substrate

because of limited research activities focusing on it. The WP contained moisture, ash, fat, protein and carbohydrates 10.61%, 13.09%, 2.44%, 11.17% and 56.02%, respectively (Al-Sayed and Ahmed, 2013). It is a suitable substrate for production of alpha amylase because of high carbohydrates content which can act as carbon sources in the SSF medium. Jackfruit is popular in few tropical countries but in some places, it is not used as food material and allowed to go waste. JS was used in this experiment since the seeds are also rich in carbohydrates which are 66.2% (Bobbio et al., 1978). It shows that JS can be used as a potential substrate for the production of enzyme. Until present, no effort has been made so far to utilize WP and JS as a substrate for alpha amylase production. Thus, the main objective of this study is to compare the capability of WP and JS to produce alpha amylase and further optimize the enzyme production using OFAT technique.

Table 2.2: Amylase production by different strain in SSF with different substrates

| Substrates | Strain | Fermentation Condition | Amylase production | References |
|---|---|---|---|---|
| Wheat bran | *Bacillus sp.* | Incubation period: 72 hours<br>pH: 6<br>Temperature: 50°C<br>Moisture content: 1:3 | 5400 U/g | (Saxena and Singh, 2011) |
| Wheat bran | *B. cereus MTCC 1305* | Incubation period: 72 hours<br>pH: 5<br>Temperature: 75°C<br>Moisture content: 1:1 | 127 U/g | (Anto et al., 2006) |
| Green gram husk | *Bacillus sp.* | Incubation period: 72 hours<br>pH: 5<br>Temperature: 75°C<br>Moisture content: 1:1 | 9824 U/g | (Prakasham et al., 2006) |
| Loquat kernels | *Penicillium expansum* | Incubation period: 6 days<br>pH: 6<br>Temperature: 30°C<br>Moisture content: 70% | 1012 U/g | (Erdal and Taskin, 2010) |
| Coconut Oil Cake | *A. oryzae* | Incubation period: 72 hours<br>Temperature: 30°C<br>Moisture content: 68% | 1827 U/g | (Ramachandran et al., 2004) |
| Banana fruit stalk | *A. niger* | Incubation period: 96 hours<br>pH: 7<br>Temperature: 40°C | 600 mg/ml/min | (Kalaiarasu and Vivekananthan, 2010) |

## 2.4 Solid State Fermentation (SSF)

SSF is defined as any fermentation process carried out on a solid material in absence of free flowing liquid. However, the substrate should require sufficient moisture to support the growth and metabolism of bacteria (Pandey, 2003). In contrast, submerged fermentation (SmF) or liquid fermentation (LF) is employed free flowing liquid substrate. The outcome of fermentation for both methods are highly dependent on different type of substrate used and type of strain used in fermentation to produce specific enzyme. SSF agricultural waste processing offers much compensation on different aspects. This is because solid state processes are less energy requirements, produce less wastewater and are environmentally friendly when solving solid waste disposal problem (Singhania et al., 2009). Furthermore, SSF has advantages over SmF such as a simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties (Saxena and Singh, 2011). Nevertheless, the major challenges on SSF method are scale up, purification of end products and biomass estimation (Singhania et al., 2009). Recently, researchers with approach of biochemical engineering have comes out with designed bioreactors that could overcome such problems that need to improved its development. SmF method is best fit for microorganisms which require high moisture content. It is primarily used in the extraction of secondary metabolites that need to be used in liquid form (Subramaniyam and Vimala, 2012). The benefits of SmF over SSF are that purification of products is easier, better control environmental factors such as pH, temperature, aeration and moisture level. SSF technique was chosen instead of SmF because of its simplicity of the fermentation process, easy control of contaminants due to low moisture level and

substrates utilized very slowly for long fermentation periods which supports the controlled release of nutrients. The major factors that affect microbial production of alpha amylase were studied; fermentation period, pH of the fermentation media, substrate concentrations and moisture content in SSF by *B. subtilis*.

### 2.4.1 Fermentation period

The result of optimum fermentation period was depending on the growth rate of species, the type of substrate used and the process itself (Krishna and Chandrasekaran, 1996). This explained the behaviors of the cell in the fermentation process. In general, enzyme production increased along with the fermentation period until reaching the maximum. The optimum fermentation period reached where the enzyme produces extensively. This is also known as exponential phase of the microorganisms (Ramachandran et al., 2004). At later stage, enzyme levels declined as the cells were denatured and lack of nutrients (Babu and Satyanarayana, 1995). Also stated by Ramachandran, 2004 it reached its stationary phase where secondary metabolites started to produce. It is important to study the fermentation period to determine the time when higher production of enzyme for commercial production of microbial products. Amylase production reached a maximum of 1012 U/g at 6th days for fungus species which is *P. expansum* (Erdal and Taskin, 2010). The optimum period of fermentation for alpha amylase production by microbial species ranges between 48-96 hours (Sivaramakrishnan et al., 2006). Correspondingly, production of alpha amylase by *B. coagulans* reached highest peak of 24 946 U/g DBB at 72 hours (Babu and Satyanarayana, 1995).

### 2.4.2 pH

It is known that the metabolic activity of bacteria is very sensitive to pH level of media. This will give an impact on the alpha amylases production which pH of media changes during fermentation due to the secretion of organic acids (Swargiari and Baruah, 2012). Thus, pH is an important factor which affects the growth and enzyme production during SSF. Bacterial species generally require an optimum pH range of 6-8 (Sivaramakrishnan et al., 2006). As Figure 2.2 shows, stated by Unakal (2012) the pH optimum for alpha amylase production using banana was by *B. subtilis.* Moreover, relatively low values of enzyme production by bacterial species were recorded at acidic conditions. However it is dissimilarity for fungus species which was reported low values enzyme production at neutral and alkaline conditions (Erdal and Taskin, 2010). The importance of determining the pH of fermentation period is to optimize the cell growth with the pH condition supplied to get high production of enzyme.

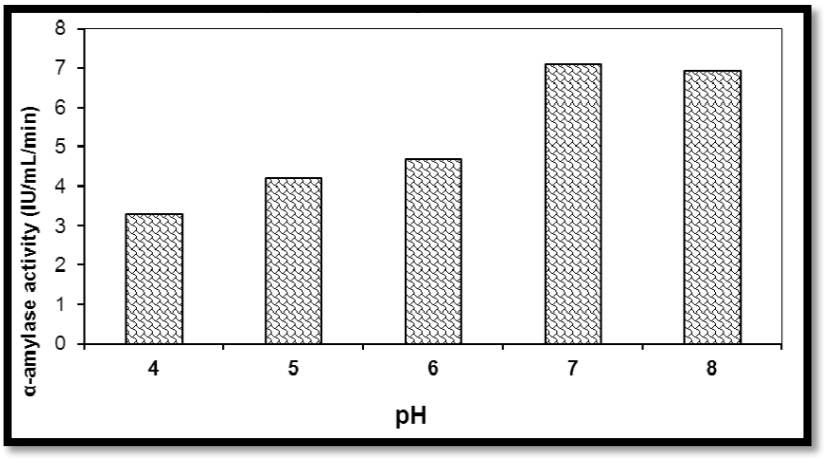


Figure 2.2: pH optimum for alpha amylase production using banana waste by *B. subtilis* (Unakal et al., 2012)

### 2.4.3 Substrate concentration

Substrate plays an important role in determining the growth of microorganisms, thereby increasing the product yield. This is because it gives physical support and nutrients for cell growth. This factor will contribute the increases enzyme production up to optimum level (Addela et al., 2015). Figure 2.3 quantified by Unakal (2012) shows that substrate concentration optimum for alpha amylase production using banana waste by *B. subtilis.* It can be seen that beyond the optimum level, as the substrate concentration increases the enzyme production will depletes. Higher substrate concentration may provide better support for attachment of microbial strain but nutrient mass transfer may be interferes during fermentation (Prakasham et al., 2006). This is said so because high concentrations of carbon sources to the strains could inhibits the enzyme synthesis (Naidu and Panda, 1998). Each substrate has their own optimum level of substrate concentration due to the composition of individual substrates. (Unakal et al., 2012)

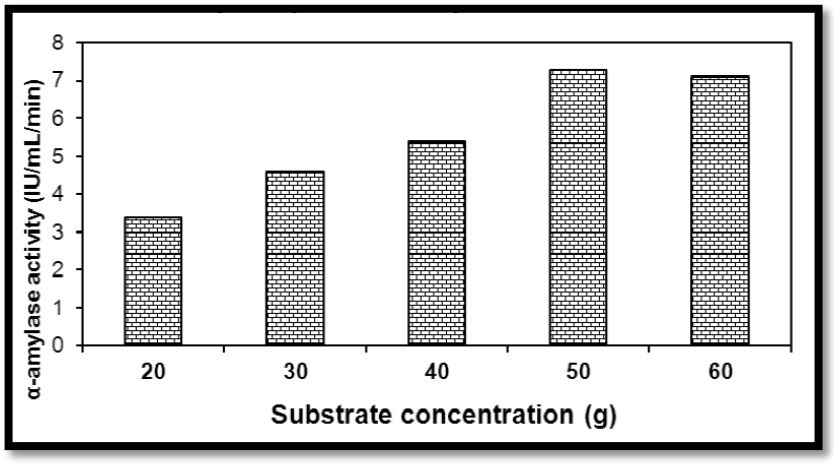


Figure 2.3: Substrate concentration optimum for alpha amylase production using banana waste by *B. subtilis* (Unakal et al., 2012)

### 2.4.4 Moisture content

Initial moisture content of SSF could influence the growth of the microorganism and thereby enzyme production (Swargiari and Baruah, 2012). The optimum moisture content for growth and substrate utilization of the fermentation depends on the organisms itself and the substrate used for cultivation. This is because initial moisture content of substrate for fermentation processes was related to availability of moisture hold by the substrates used. Higher moisture content reduces the porosity of the substrate, thereby reducing the gas exchange (Baysal et al., 2003). This will interfere the oxygen transfer for cell growths thus decrease the enzyme secretion. Lower moisture content cause to sub-optimal growth and reduces solubility of the nutrients of the substrate which is also decreases enzyme production (Saxena and Singh, 2011). The optimum initial moisture content for bacterial species was reported to require in range of 70-80% (Sivaramakrishnan et al., 2006). Maximum alpha amylases produce using *B. subtilis* stated by Baysal et al. (2003) was 30%. In addition, nature of the moistening agent also determines the enzyme production (Swargiari and Baruah, 2012). For an example, salt solution (Babu and Satyanarayana, 1995), phosphate buffer (Lonsane and Ramesh, 1990) and many more. Hence, effect of moisture content was studied as well to achieve high enzyme production.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Microorganism

*B.subtilis* was obtained from stock cultures at the School of Chemical Engineering Campus, USM. The microorganism which was maintained on nutrient agar was used in this research.

## 3.2 Preparation of substrate

The WP and JS were obtained from the nearby market or dumping area. The peels and seeds were washed thoroughly and cleaned to remove any unwanted dirt and inorganic matters on its surface. The peels were sliced into smaller size, about 0.2-0.4 cm to facilitate the drying process, which was dried in an oven (Krishna and Chandrasekaran, 1996) at temperature of 50°C for 24 h to remove all the moisture contents. Same procedure was performed on JS but was dried at temperature of 60°C for 24 h. The dried substrates were then ground using mixer grinder and stored in an air tight container at room temperature (Unakal et al., 2012). Figure 3.1 shows the dried WP after 24 h at 50°C and Figure 3.2 shows the grinded WP. Whereas, Figure 3.3 shows the dried JS after 24 h at 60°C and Figure 3.4 shows the grinded JS.

Figure 3.1: Dried WP after 24 h at 50°C

Figure 3.3: Dried JS after 24 h at 60°C

Figure 3.2: Grinded WP

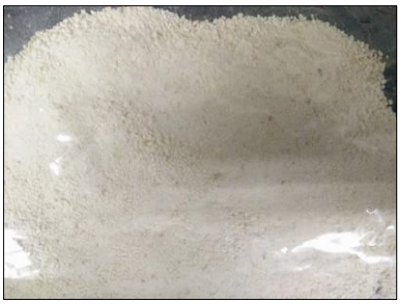

Figure 3.2: Grinded JS

### 3.3 Preparation of Inoculum

*B. subtilis* strain from stock culture was transferred to a 250 mL conical flask containing 100 mL of nutrient broth in laminar air flow cabinet, and the cells were then incubated at temperature 35°C and kept on shaker at 150 rpm (Unakal et al., 2012) to get a standardized inoculum. The exponential phase of *B. subtilis* is around seventh or eighth hour (based on the growth curve in Figure A.1 Appendices) thus the cells were harvested from nutrient broth after 7 or 8 hour of incubation with cell concentration at 0.4 g/L and OD is 0.9-1.0. The cells in nutrient broth were centrifuged to get cell pellet. Then it was re-suspended with sterile distilled water and ready to be used for SSF.

### 3.4 Solid State Fermentation (SSF)

The inoculated cells (10 mL) were centrifuged at 4000 rpm for 10 min. The re-suspended cell pellet was added to the 250 mL Erlenmeyer flasks containing 10 g of WP. Fermentation media (10 mL) from Unakal et al. (2012) with slight modification comprised of $MgSO_4.7H_2O$ (0.2 g/L), $CaCl_2$ (0.02 g/L), $KH_2PO4$ (1.5 g/L), $NH_4NO_3$ (1.5 g/L), $FeCl_3$ (0.05 g/L) and glucose (5g/L). The pH of the media was adjusted using 0.1-N hydrochloric acid (HCl), acidic or 0.1-N sodium hydroxide (NaOH) and also was sterilized in autoclave. WP was added to the medium served as the source of carbon and the samples aseptically withdraw periodically and assayed for amylase activity.

### 3.5 Optimization studies using OFAT

OFAT is one of the optimization methods which vary only one factor or variable at a time while keeping others fixed. There are three independent parameters studied for the alpha-amylase production which are fermentation period (h), pH of the medium, substrate concentration (g) and moisture content (w/v %). These parameters would influence the enzyme yield during SSF will be optimize over a wide range. The approach is to optimize each parameter independently of the others and optimal conditions will be employed in subsequent experiments. In sequential order the various process parameters were optimized for maximal enzyme production: fermentation period (24-96 hrs), pH of the medium (5-8), substrate concentration (8-14 g) and moisture content (52-93 %). For moisture content, each substrate concentration was calculated its initial moisture content (w/v %) by varying the weight of the substrate and the volume of growth medium mineral and cells was constant (15 ml).

### 3.6 Enzyme Analysis

#### 3.6.1 Glucose standard Curve Development

Glucose solutions were prepared with varying concentrations of 0.1, 0.3, 0.5, 0.7, 0.9 g/L. Then, the dinitrosalicyclic (DNS) acid reagent solution (1%) was prepared using dinitrosalicyclic acid (2.50 g), sodium sulphite (0.125 g) and sodium hydroxide (2.5 g) in 200 mL of distilled water and the solution was then topped up to 250 mL of flask. Potassium sodium tartrate (40% v/v) was prepared by adding 40 g of potassium sodium tartrate to 50 mL of distilled water and the solution was topped up to 100 mL of flask. DNS acid reagent (3 mL) was mixed with every 3 mL of glucose sample in test tube (with different concentrations). The mixtures were boiled at 90°C for 10 min to develop a red-brown color. After cooling to room temperature, the absorbance at 575 nm wavelength was measured using UV-Vis Spectrophotometer. The OD versus glucose concentration (g/L) was plotted (Figure A.2 Appendices). This graph was used as a reference or standard for the calculation of enzyme activity.

#### 3.6.2 Enzyme Extraction

Enzyme alpha-amylase from solid substrate was extracted by mixing 50 mL of 0.1M phosphate buffer pH 7 and shake in incubator shaker at 250 rpm for 30 min. The buffer containing enzyme was separated from solid substrates through filter paper. The filtrate was centrifuged at 4000 rpm for 10 min. The clear brown supernatant was used as the enzyme assay analysis.

### 3.6.3 Alpha Amylase Enzyme Assay

Alpha-amylase activity was determined by the procedure of Bernfeld (1955) using soluble starch as a substrate. The reaction mixture containing 200 µL of 1% (w/v) in 300 µL 0.1M phosphate buffer pH 7 and 150 µL of enzyme solution was incubated at 37°C for 30 min. The reaction was stopped by adding 400 µL of 3,5-dinitrosalicyclic (DNS) acid solution followed by heating in a boiling point for 5 min and cooling at room temperature. Then, distilled water was added until the solution volume was 12 mL. The initial reading (blank) was prepared by boiling 800 µL of enzyme solution for 20 min to denature the enzyme protein structure and cooling at room temperature. Then distilled water was added until volume 12 mL. Absorbance of each solution was measured at 489 nm using a UV-Visible spectrophotometer.

### 3.6.4 Enzyme activity calculation

Alpha-amylase activity will be determined by the spectrophotometric method in an assay mixture containing enzyme extract, starch as substrate and DNSA (3, 5 dinitro salicylic acid) as coupling reagent. One Unit of enzyme activity is defined as the amount of enzyme, which releases 1 µmole of reducing sugar as glucose per minute under the assay conditions. The absorbance (optical density, OD) was read at 540 nm using a Spectrophotometer against glucose as the standard (Saxena and Singh, 2011). The formula of enzyme activity was given by

$$\frac{U}{g} = \frac{\Delta \mathrm{OD} \times D}{W \times t \times V}$$

Where ΔOD is the difference in OD which referred to the glucose standard curve in μmol/L. *D* is the dilution factor in this experiment and *W* is the mass of WP substrate. *t* is the incubation time and *V* is the sample volume in mL.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 Evaluation of substrates for alpha amylase production

Two types of substrate were preliminary studied which are watermelon peels (WP) and jackfruit seeds (JS). These wastes were carefully chosen on this experiment on the basis of hydrocarbon content which is carbon sources to the *B. subtilis* in SSF. As shown in Figure 4.1, using WP as a substrate gives higher production of enzyme alpha amylase compared to JS. The carbohydrates content for WP and JS are 56.02% (Al-Sayed and Ahmed, 2013) and 66.2% (Bobbio et al., 1978) respectively. According to Mahanta and Kalita (2015), the carbohydrates content in JS is a type of polysaccharide also known as starch. As reported in literature, *Bacillus sp.* is well known to produce alpha amylases in starch medium such as wheat bran (Babu and Satyanarayana, 1995), cheese whey (Baysal et al., 2003) and rice husk (Sodhi et al., 2005) in SSF. Although the carbohydrates content for JS was higher than WP but the suitable type of microbial used for the substrate and its environment conditions has also influenced the microbes to utilize the nutrient supplied to produce higher enzymes. Soluble sugars such as fructose, glucose and sucrose are the major sugars present in WP. These sugars types supported good growth and alpha amylase production (Rajagopalan and Krishnan, 2008). The enzyme activity for WP was higher compared to JS in this preliminary experiment which are 370.29 U/g and 267.38 U/g correspondingly, thus WP was selected as substrate for further optimization study.