Production and Characterization of Amylase Produced by Raw Starch Degrading Bacteria Using Submerged Liquid Fermentation (SmF) of Agricultural Waste

Nurnikma binti Tuah (19589)

Bachelor of Science with Honours
(Resource Biotechnology)
2010
DECLARATION

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

............................................

(NURNIKMA BINTI TUAH)

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak
ACKNOWLEDGEMENT

First of all I would like to express my thankfulness to Allah S.W.T that I would be able to complete STF3014 Final Year Project with fulfilled the Resource Biotechnology program course as a undergraduate student of University Malaysia Sarawak, UNIMAS.

I would like to expressed my sincere thanks to my supportive supervisor, Dr. Awang Ahmad Sallehin bin Awang Husaini for his trust, help and guidance during the this final year project was conducted. I also would like to thanks to all the laboratory assistants and postgraduate students of Molecular Genetic Laboratory (MGL) of Faculty of Resource Science and Technology (FRST) UNIMAS for their great support, ideas and knowledge sharing.

Thanks to all my fellow friends for their supporting and helps during the completion of the project. I also want to say thanks to the entire fellow who in directly and indirectly involved in my project from the start until the final report submission.

Finally, I would like to thanks to my parent and family for their support and encourage me for what I have done.

Thank you very much.
# TABLE OF CONTENTS

Acknowledgement ........................................................................................................... I

Table of Contents ............................................................................................................... II

List of Abbreviation........................................................................................................... V

List of Tables and Figure................................................................................................. VI

Abstract ........................................................................................................................... 1

*Abstrak* .......................................................................................................................... 1

1.0 Introduction................................................................................................................... 2

2.0 Literature review........................................................................................................... 5
   2.1 Microorganisms species............................................................................................ 5
   2.2 Carbon source........................................................................................................... 5
       2.2.1 Potato waste ..................................................................................................... 6
       2.2.2 Cassava waste .................................................................................................. 6
   2.3 DNS assay method.................................................................................................... 7
   2.4 Parameter optimization of enzyme production...................................................... 7
       2.4.1 Time of incubation .......................................................................................... 8
       2.4.2 Temperature ................................................................................................... 8
       2.4.3 pH .................................................................................................................. 8
   2.5 Characterization of enzyme activity........................................................................ 9
       2.5.1 Effect of pH on enzyme activity ................................................................. 9
       2.5.2 Effect of temperature on enzyme activity ................................................. 9
   2.6 Kinetic Study............................................................................................................. 10

3.0 Materials and method................................................................................................. 11
   3.1 Laboratory preparation.............................................................................................. 11
       3.1.1 Microorganisms preparation ........................................................................ 11
       3.1.2 Growth medium preparation .......................................................................... 12
       3.1.3 Iodine solution .............................................................................................. 12
3.1.4 DNS reagent..............................................................................12
3.1.5 Rochelle salt.............................................................................13
3.1.6 Glucose standard curve............................................................13
3.1.7 Substrate preparation...............................................................13
3.2 Amylase production screening test .............................................14
3.3 Fermentation experimental design ............................................15
3.4 Enzyme assay..............................................................................15
3.5 Parameter optimization of enzyme production.........................16
  3.5.1 Carbon source..........................................................................16
  3.5.2 Incubation period.................................................................16
  3.5.3 Temperature...........................................................................16
  3.5.4 pH.........................................................................................17
  3.5.5 Substrate ratio.........................................................................17
3.6 Parameter optimization on the effect of enzyme activity............18
  3.6.1 Time of incubation...............................................................18
  3.6.2 pH.......................................................................................18
  3.6.3 Temperature..........................................................................19
3.7 Kinetic Study..............................................................................19
4.0 Result and discussion..................................................................20
  4.1 Starch degrading bacteria confirmation test.............................20
  4.2 Parameter optimization of enzyme production........................22
    4.2.1 The incubation period for α-amylase production..................22
    4.2.2 Effect of temperature on enzyme production......................24
    4.2.3 Effect of pH on enzyme production.....................................26
    4.2.4 Effect of substrate ratio on enzyme production...................28
  4.3 Amylase enzyme characterisation............................................29
    4.3.1 Time of α-amylase incubation............................................30
    4.3.2 Effect of temperature on enzyme activity............................31
    4.3.3 Effect of pH on enzyme activity..........................................33
  4.4 Kinetic study of α-amylase.......................................................34
5.0 Conclusion and recommendation................................................37
6.0 References........................................................................................................38

7.0 Appendices........................................................................................................41
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>[S]</td>
<td>Substrate concentration</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalicyclic Acid</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>IU</td>
<td>One International Unit</td>
</tr>
<tr>
<td>K_m</td>
<td>[S] of 1/2 V_max</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Mililiter</td>
</tr>
<tr>
<td>MSM</td>
<td>Mineral Salt Medium</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
<tr>
<td>SmF</td>
<td>Submerged Liquid Fermentation</td>
</tr>
<tr>
<td>SSS</td>
<td>Solid-state fermentation</td>
</tr>
<tr>
<td>V_max</td>
<td>Maximum velocity at enzyme saturating [S]</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

Table 1  
Bacillus sp. in α-Amylase Production………………………………………5

Table 2  
Glucose Concentration for Glucose Standard Curve…………………..13

Table 3  
Bacteria Culture on Plate Agar containing MSM and 1% Soluble Starch...14

Table 4  
Substrate Ratio for Submerged Liquid Fermentation…………………17

Table 5  
Volume of Enzyme, Substrate and Buffer for Kinetic Study…………19

Table 6  
Result of α-amylase Kinetic Study……………………………………34

Figure 1  
Clear site on Plate A and B of D3 colonies……………………………20

Figure 2  
Incubation period for enzyme production……………………………22

Figure 3  
Effect of temperature on α-amylase production………………………24

Figure 4  
Effect of pH on enzyme production……………………………………26

Figure 5  
Effect of substrate ration on enzyme production……………………28

Figure 6  
Effect of time incubation on α-amylase activity………………………30

Figure 7  
Effect of temperature on enzyme activity……………………………31

Figure 8  
Effect of pH on enzyme activity…………………………………………33

Figure 9  
Lineweaver-Burk Plot…………………………………………………35

Figure 10  
The Lineweaver-Burk plot of the kinetic study……………………35
Production and Characterization of Amylase Produced by Raw Starch Degrading Bacteria Using Submerged Liquid Fermentation (SmF) of Agricultural Waste

NURNIKMA BINTI TUAH

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SARAWAK
2010
Production and Characterization of Amylase Produced by Raw Starch Degrading Bacteria Using Submerged Liquid Fermentation (SmF) of Agricultural Waste

Nurnikma binti Tuah

Resource Biotechnology Programme
Faculty of Science and Technology
University Malaysia Sarawak

ABSTRACT

Amylase is one of the most important enzymes in industrial application. The study investigates the production and the characterization of the amylase that was extracted from raw starch degrading bacteria. D3 isolate is the best amylase producer among three types of bacterial culture. Potato is the best substrate for inducing amylase enzyme production. The result shows the highest production of amylase (2.0179 IU/mL) detected at temperature 37°C after 15 hours incubation period. The best pH for amylase production is pH7 with activity 1.0034 IU/mL as it is the best condition for the bacterial growth. The enzyme was analyzed by using dinitrosalicylic acid (DNS) assay method. The characterization of enzyme activity was shown the highest activity (0.4698 IU/mL) was produced at temperature 32°C after 20 minutes incubated with 1% soluble starch. The best pH for the enzyme activity is pH5 with 1.3942 IU/mL of reducing sugar detected. The $V_{max}$ value of enzyme kinetic study is 0.3429 mg/mL/min and the $K_m$ is 1742.4554 mg/mL.

Key words: D3, amylase, DNS, enzyme activity, kinetic study.

ABSTRAK

Amilase merupakan salah satu enzim yang penting dalam aplikasi industri. Kajian ini menyelidik penghasilan dan sifat enzim amilase yang dihasilkan oleh bacteria yang boleh menguraikan kanji. Jenis bakteria D3 telah menunjukkan penghasilan enzim amylase dengan lebih cepat berbanding dua jenis bacteria yang lain. Kentang dipilih sebagai sumber karbon yang paling banyak menghasilkan enzim (2.0179 IU/mL ) tersebut pada suhu 37°C selepas 15 jam pemerapan. pH 7 merupakan medium yang sesuai untuk menghasilkan enzim dengan aktiviti 1.0034 IU/mL. Gula yang terhasil daripada proses fermentasi dianalisis dengan menggunakan acid dinitrosalicylic. Sifat enzim amilase yang yang dihasilkan telah menunjukkan aktiviti yang paling tinggi (0.4698 IU/mL) pada suhu 32°C selepas 20 minit bertindak balas dengan 1% kanji. Manakala, enzim tersebut aktif dalam pH 5 dengan bacaan 1.3942 IU/mL gula yang dihasilkan. Nilai $V_{max}$ daripada profil kinetik adalah 0.3429 mg/mL/min dan $K_m$ ialah 1742.4554 mg/mL.

Kata kunci: D3, enzim amilase, DNS, aktiviti enzim, kinetic enzim.
1.0 INTRODUCCION

Amylase is one of the most important industrial enzymes which hydrolyze starch molecule to give diverse products including dextrins and smaller polymer composed of glucose units. These enzymes are important in biotechnology application such as food, fermentation, and paper industry. Amylases can be produced by plants, animals, fungi and microorganisms.

Amylases can be classified into three types, α-amylase (E.C. 3.2.1.1), β-amylase (E.C. 3.2.1.2) and glucoamylase (E.C. 3.2.1.3). Alpha amylase degrade starch molecule by hydrolyzing α-1, 4-glycosidic bond in polysaccharides and produce α-anomeric arrangement. This type of amylase require calcium ion for stability, structural integrity and activity which is also called metalloenzymes (Sivaramakrishnan et al., 2006). Beta amylase also known as glycogenase hydrolyzes the second α-1, 4-glycosidic bond by producing disaccharide compound which is maltose. Glucoamylase which is also known as γ-amylase will hydrolyze last α-1, 4-glycosidic bond to produce amylase and amylopection while the α-1, 6-glycosidic linkage hydrolyze in order to produce glucose. Glucoamylase prefer acidic environment with optimum pH 3. The main target for this research is production alpha amylase and its characteristics. Debranching enzymes will catalyze α-1, 6 bond and produce long linear polysaccharides while transferases will transfer donor molecule to glycosidic acceptor to form new glycosidic bonds (Maarel et al., 2002).

Nowadays, as the petroleum-derived fuel and industrial feedstocks has been decrease and the fuel or gas derived from renewable sources is one of the solutions to avoid this problem. Bioethanol is by-product of fermentation process which is produced from starch degraded into lactose and the sugar will ferment into alcohol. This alternative fuel derived from food waste can reduce the air pollution because it is clean-burning (Kumar et al., 1998). The waste from food industrial cause the pollution and it needs the
better waste management system. So, the utilizing all these food waste to produce ethanol will reduce the environment pollution and that generated fuel can be reused by the industry as a biofuel or biogas. Another application of α-amylase is the uses in bread and baking industry and act as an anti staling agent. α-amylase widely used in bread and rolls to provide the higher volume, better colour and more softer.

In this study, bacteria were used to produce amylase enzyme by submerged liquid fermentation in shake flask and raw starch as a substrate to induce the enzyme production. The main advantage of using the bacteria in this system is the economical aspect of bulk production of microbes and easy to manipulate to obtain enzymes with desired characteristic. The examples of microorganisms reported which produced α-amylase were *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens* and *Bacillus stearothermophilus* (Sivaramakrishnan et al., 2006). The substrate sources for induction of the α-amylase were cassava waste and potato waste. The amount of enzyme produce from each raw starch sources was compared and the highest enzyme productions from the substrate were further used for enzyme production, optimization, purification and characterization of α-amylase produced. After that, the kinetic was performed to analyze the different amount of substrate that can react with enzyme at optimum temperature and pH.

The objective of experimental study is to;

- Identify the best amylase producing bacteria from the screening test.
- Investigate the optimization parameter for enzyme production by using submerged liquid fermentation.
• Identify the characteristic and kinetic study of α-amylase enzyme produced from raw starch degrading bacteria.

The hypothesis of this study was that bacteria will grow in prepared medium. The screening test with iodine solution will produce clear halos indicating that the starch has been degraded. The selected bacteria will be appeared in purple colour after Gram staining experiment which is indicates the Gram-positive bacteria. Bacteria produce amylase with highest quantity at pH7-7.5, temperature 37°C and the time for incubation 15 hours. Amylase produces larger amount of glucose from starch compound at temperature 90°C and pH 4.5 (Riaz et al., 2003).

Since, there are three types of bacterial culture provided which are 5K, D3 and H1, the best type that have the capability to produce highest amylase quantity among these three type will be selected after screening test. However, all three types of bacterial culture are able to produce amylase to degrade starch molecule, only the best will be used to further fermentation process. The selection of these bacteria carried out by screening test. The bacteria culture spread onto agar containing Minimal Salt and soluble starch. The agar plate screened with iodine solution after overnight incubated. The screening test is very important because this provide the initial confirmation of the suitable type of bacteria that have the highest capability in producing amylase to degrade starch.
2.0 LITERATURE REVIEW

2.1.0 Microorganisms species

*Bacillus* sp. is usually used in the research before in order to determine the best raw starch degrading bacteria in industrial aspect because of the thermo stability characteristic is important. *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens* and *Bacillus stearothermophilus* are the best bacterial species producing that was reported in the recent studies (Sivaramakrishnan *et al.*, 2006). In order to produce thermotolerant amylase, the bacterial and actinomycetes amylases were identifying to have thermostability properties. The production of amylase from food waste by actinomycetes are not much good turn compared to α-amylase produced by bacterial species (Singh *et al.*, 2008).

**Table 1**: *Bacillus* sp. in α-Amylase Production

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>Type of substrate</th>
<th>α-amylase produce</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Rice husk</td>
<td>21 760 U/g</td>
<td>(Baysal <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>Potato peel</td>
<td>270 U/mL</td>
<td>(Shukla and Kar. 2006)</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>Soluble starch</td>
<td>72 U/mg</td>
<td>(Gangadharan <em>et al.</em>, 2008)</td>
</tr>
</tbody>
</table>

2.2.0 Carbon source

Substrate source composed of carbon compound is essential to induce the α-amylase production. Most reports suggest maltose is suitable starch used for induction of α-amylase by *Aspergillus oryzae*. That was also reported by JA (1984) shows the increasing 20 folds of enzyme activity when maltose and starch were used as an inducer in *Aspergillus oryzae*. However the agriculture wastes are the best decision in order to reduce the pollution of environment. Agriculture wastes such as potato waste and cassava waste (Abu *et al.*, 2005) are examples of the agro-processing by products have been used to induce enzyme production in the bacterial. The highest of α-amylase produced by bacteria...
when using the wheat bran as a substrate for induce the enzyme production (Balkan and Ertan, 2006).

2.2.1 Potato waste

The potato waste can contribute to the waste water problem if the amount of the potato industry processing is larger. There are three types categories of waste water problem related to potato industry.

1. Peeling equipment for hospitals, prison and barrack,
2. Industry of potato processing
3. Potato starch mills.

The main source of waste water pollution is the peeling stage during potato processing when large quantity of peel discharge into water stream (Peters, 2009). The skins of pre-washed tuber of potato were removed by steaming and then remove the skin itself. After that, the trimming and slicing process were produce waste thus cause the after washer seriously polluted by increasing in Biological Oxygen Demand (BOD). The manufacture of potato starch produces solid potato waste as the by-product of processing. This solid potato waste has the starch remained and can be used for fermentation process in order to produce industrial enzyme such as amylase (Peters, 2009).

2.2.2 Cassava waste

Cassava is a crop plant that grown in different range production system. Cassava containing various mineral needed for growing such as carbohydrate, phosphorus, calcium, magnesium and nitrogen (Cereda and Takahashi, n.d.). Cassava waste is residue of plant after processing. There are four types of wastes produced from cassava processing to produce starch; outer skins, inner rinds, fibrous residues and waste water (Ray and Ward, 2006). The solid waste from cassava produced from flour producing industry and produce liquid residue during starch processing. The waste was generated from the cassava root.
Some other solid wastes generated from cassava root are crude bran, inner peel, bran, baggase and brown peel. However, these solid residues of cassava can damage the environment if it does not properly handle and managed.

Cassava waste can be used in various way in order to reduce the effects toward environmental. Some of the implementation are, used the cassava waste as animal feed and process into agriculture fertilizers. Besides that, cassava also used to produce biomass such as yeast *Trichosporon* sp.. The fermentation process of cassava waste as a substrate to induce the enzyme production by microorganism is one of the application of cassava waste residues manage.

### 2.3.0 DNS assay method

The Dinitrosalicylic acid reagent was developed by Summer and his colleague (1921) to determine the amount of reducing sugar. The DNS reagent is composed of sodium hydroxide, dinitrosalicyclic acid, phenol, sodium sulphite and Rochelle salt. The uses of the Rochelle salt in DNS method is to prevent the oxidation of the reducing sugar. Phenol was used to enhanced the colour of the reaction and sodium sulphite was used to stabilize the colour obtain after boiling step. However, the major problem in using DNS method is the lost of reducing sugar during the test being analyzed (Summer, 1944). The study was done by Miller (1959) in order to investigate the effect of each component of the reagent used.

### 2.4 Parameter optimization of enzyme production

Optimization step in enzyme production is important in order to determine the optimum level where the enzyme is highly produced during the fermentation. The examples of optimization parameter that widely used in experimental are time of incubation, temperature, pH, and substrate ratio.
2.4.1 Time of incubation

Riaz et al., (2003) indicates the maximum \( \alpha \)-amylase produced from *Bacillus subtilis* is 535IU/mL/min at pH 7.5, 40°C after 48 hours of incubation period. Other study that was done by Panda et al., (2008) indicates that a linear increase of \( \alpha \)-amylase production when incubation period up to 36 hours. After that, the production of \( \alpha \)-amylase from lactic acid bacteria was declined as the time interval increase.

2.4.2 Temperature

Mostly the temperature for \( \alpha \)-amylase production is related to the growth of the organisms itself. Commonly, \( \alpha \)-amylase was produced at much wider range of temperature among the bacteria species. Castro et al. (2003) has been reported that \( \alpha \)-amylase was produced at 36°C by *B. amyloliquefaciens*. *Bacillus* sp. was capable to produce amylase at the range 28-52°C. The production will decline when the temperature increase starting from 37°C since at this point the production is maximum (Sudharhsan et al., 2007). However, hyperthermophiles bacteria, *Thermococcus profundus* also have been reported produce amylase at 80°C (Chung et al., 1995). A cold active \( \alpha \)-amylase from Antarctic psychrophile, *Alteromonas haloplanktis* was reported by Feller et al., (1998) to produce maximum a-amylase at 4°C

2.4.3 pH

pH is one of the important factor for growth condition of bacteria because they are sensitive to hydrogen ion concentration that present in the medium. Mostly bacteria required neutral pH for their optimum growth thus for enzyme production. Bacteria species such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* require pH7 for enzyme production at optimum level (Tanyildizi et al., 2005). The hyperthermophilic archae such as *Thermococcus profundus* produce optimum level of \( \alpha \)-
α-amylase at pH5 (Vieille and Zeikus, 2001). Thermophilic anaerobic bacteria *Clostridium thermosulfurogenes* was reported by Swamy and Seenayya (1996) capability to produce α-amylase at pH7.

### 2.5 Characterization of enzyme activity

α-amylase characteristic such as thermostability and pH value should match with industrial demands. Mostly the industrial needs enzyme that can react at different temperature and pH optima for enzymatic processes that involve two reaction steps which are liquefaction and saccharification (Abd Aziz, 2002).

#### 2.5.1 Effect of pH on enzyme activity

The general pH optima of α-amylase activity are varying from 2 to 12 (Vihinen and Mantsala, 1989). The study done by Xu and Yan (2007) indicates that the enzyme was optimally active at pH 5.0 and more than 90% of the peak activity was shown in the pH range from 4.5 to 6.0.

#### 2.5.2 Effect of temperature on enzyme activity

The optima temperature for amylase activity is equal to the growth condition of the bacteria. The lowest optima temperature is 25-30°C for amylase produced by *F. oxyporum* (Chary and Reddy, 1985) and the highest is from archaeabacteria which is about 100-130°C *Pyrococcus furiosus* and *Pyrococcus woesei* (Fogarty and Kelly, 1979). Yang and Wang (1999) were reported in their study that the highest activity of enzyme produced by *Streptomyces ramous* between 35 and 50°C and lost of activity when the temperature higher than 60°C.
2.6 Kinetic study

The study done by Al-Qodah et al. (2007) to determine the kinetic parameter of α-amylase produced by Bacillus sphaericus from batch fermentation shows the kinetic parameters of α-amylase activation $V_{\text{max}}$ and $K_m$ are 263 µmole mg$^{-1}$ enzyme min$^{-1}$ and 0.97 mg/ml.
3.0 MATERIALS AND METHOD

3.1.0 Laboratory preparation

3.1.1 Microorganisms preparation

The 10 µL of stock culture of 5K, D3 and H1 were subcultured into autoclaved bijou bottle containing Luria Broth (LB). LB was prepared in order to grow the bacteria culture. The LB was prepared by dissolved 10g of Luria Broth (SIGMA) powder into 400 mL distilled water. LB was used as media to do the bacterial subculturing in the bijou bottle. After that, the LB was autoclaved at 121°C for 1 hour to kill all the possible contamination from other living organism. After autoclaved, LB was left to cool down at room temperature. Three types of bacteria were overnight incubated at 37°C with shaking. After overnight incubation, 1 mL of the bacterial cultures was pipetted into 1.5 mL autoclaved centrifuge tube. The sub-culturing activity was done every 2 days. The inoculation activity was carried out in laminar flow hood in order to ensure the aseptic was carried out during the inoculation.

In order to prepare glycerol stock of bacteria, 40% of glycerol had to be prepared. The final volume of 40% glycerol was 10 mL. 4 mL of pure glycerol was added into 6mL of distilled water followed by autoclaving. After overnight incubation, 1mL of all three types of bacteria culture 5K, D3 and H1 was pipetted into autoclaved 1.5 centrifuge tube. Then, 0.5 mL of 40% glycerol solution was added into each centrifuge tubes. Eppendorf tubes containing glycerol stock cultures were snap frozen into liquid nitrogen and then kept in freezer at -20°C.
3.1.2 Growth medium preparation

The Minimal Salt Medium was prepared for 1 L of distilled water. Then, 0.5g KH$_2$PO$_4$, 0.5g (NH$_4$)$_2$SO$_4$, 0.5g MgSO$_4$.7H$_2$O were added into the distilled water. Then, the media was autoclaved for sterilization process to kill other microorganisms that exist in the media before further used for bacteria growth. Minimal Salt Medium (MSM) plus agar was used for the screening experiment. MSM containing 0.2g KH$_2$PO$_4$, 0.2g (NH$_4$)$_2$SO$_4$, 0.2g MgSO$_4$.7H$_2$O, 6g agar powder and 4g soluble starch. Then, it was dissolved in 400mL distilled water. The medium was adjusted to neutral pH which is 7. Then, the mixture was autoclaved and poured into Petri dish to allow the MSM agar solidified and cool.

3.1.3 Iodine solution

Iodine solution had to be prepared before doing the screening test for amylase production. Starch will forms a black blue when complexes with iodine (Hollo and Szeitli, 1968). The blue colour will changed into yellowish colour when starch hydrolyzes by amylase produced by bacteria (Gupta et al., 2003). Iodine solution was prepared by adding 0.1g crystal iodine into 30mL distilled water. After that, 0.2g of potassium iodide added and the component was stirred until the particles completely dissolved. Iodine solution then put into aluminum wrapped bottle because it sensitive to light intensity.

3.1.4 DNS reagent

The DNS assay (dinitrosalicyclic acid) method used to determine the amount of reducing sugar. DNS reagent was prepared by dissolving 2 g dinitrosalicyclic acid (DNSA), 0.4 g phenol, 0.1 g sodium sulphite and 2 g NaOH into 200 mL distilled water. The mixture was stirred until all the particles completely dissolve. DNS reagent was then kept in aluminium wrapped bottle because it is sensitive toward light intensity.
3.1.5 Rochelle salt

Enzyme assay activity also required Rochelle salt also known as potassium sodium tartarate solution. Rochelle salt was prepared by dissolving 80 g of potassium sodium tartarate tetrahydrate into 200mL of distilled water. Then, Rochelle salt was kept in refrigerator.

3.1.6 Glucose standard curve

The glucose standard curve was prepared in order to estimate the amount of glucose produced by bacteria during the fermentation process. This analysis was carried out by DNS assay method with different known concentration of glucose solution. Table 2 below showed the glucose concentrations that were used to prepare the standard curve. The glucose stock solution used was 2 mg/mL.

Table 2: Glucose Concentration for Glucose Standard Curve

<table>
<thead>
<tr>
<th>[Glucose] (mg/mL)</th>
<th>Stock (mL)</th>
<th>Distilled Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>0.4</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>0.6</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.2</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>1.4</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>1.6</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>1.8</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.1.7 Substrate preparation

The substrates used in the submerged liquid fermentation were potato and cassava waste. Potato and cassava were washed with water to remove the impurities. After that, peeling process was done to remove the peel from potato and cassava. Both substrates were
washed before scrapped. Potato and cassava were scrapped into smaller particles. After that, the water was removed by sieving and potato and cassava waste were collected which is also known and “hampas”. Potato and cassava waste were separately dried overnight in the oven at 65°C to remove water residue. Then, both wastes were kept in plastic container at room temperature for use in fermentation process. The potato and cassava waste were selected to be used in the experiment because it already available in market and the waste itself are contained starch molecule that induced amylase production by bacteria.

3.2 Amylase production screening test

The screening test for the amylase production by three types of bacterial isolates named: 5K, D3 and H1 were carried out by using MSM agar plus 1% soluble starch. Triplicate was done for each type of bacteria at different inoculums volume. The bacteria were spread onto the MSM agar according to the following table;

<table>
<thead>
<tr>
<th>Type</th>
<th>Plate</th>
<th>A (µL)</th>
<th>B (µL)</th>
<th>C (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5K</td>
<td></td>
<td>100</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>D3</td>
<td></td>
<td>100</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>H1</td>
<td></td>
<td>100</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

All bacteria cultures were incubate at 37°C for 2 days. After incubation period, all of the plates containing bacteria were screened by using iodine solution to determine the capability each types of bacteria to produce amylase to degrade the soluble starch.
3.3 Fermentation experimental design

According to Khan and Husaini (2006), the experiment was carried out by using Enlenmeyer Flasks (100mL) containing 20mL fermentation medium that inoculated with overnight bacteria culture. The shaker flasks were done by duplicates for each parameter to increase the accuracy of the result. The enzyme analysis was carried out in duplicates for each the replicates of the parameter. There are four data were collected from each the parameter experiment. The control was setup for each. The enzyme extraction was carried out by using Whatman No. 1 filter paper and the filtrates will be centrifuged at 13 000 rpm to obtain the crude enzyme preparation.

3.4 Enzyme assay

The DNS assay (dinitrosalicylic acid) method was used to determine the amount of reducing sugar and compared with glucose as the standard (Bernfeld, 1995). The enzyme assay was carried out by adding 400 µL of crude enzyme with 1% (w/v) soluble starch into 20 mL tube. Then, the mixture was incubated for 10 minutes at 37°C. After that, the reaction was stopped by adding 800 µL of DNS reagent into the enzyme mixture and boiled for 15 minutes. Approximately 400 µL of Rochelle salt was added into the mixture right after the boiling process. The tube was then vortexed to homogenize the mixture content. Lastly, the mixture was read by using spectrophotometer at O.D. 575nm. The blank for enzyme assay was done by replacing 400µL crude enzyme with 400 µL distilled water.

Besides that, the initial glucose in each of the fermentation parameters were also measured. The initial glucose procedure is similar as the enzyme assay procedure but the 400 µL of 15 soluble starches was replaced with 400 µL of distilled water. The sample was read by using spectrophotometer at O. D. 575nm. One unit of amylase activity was defined as