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# Effect of Nutritional Factors on Amylase Production from Isolate M

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# DECLARATION

I declare that this thesis entitled “Effect of Nutritional Factors on Amylase Production from Isolate M” is the results of my own research except as cited in the references.

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## ABSTRAK

Amilase adalah sejenis enzim hidrolisis yang digunakan secara meluas dalam pelbagai aplikasi atau industri, termasuk industri makanan, pembuatan bir, tekstil, kertas, dan biofuel. Amilase diperlukan dalam kuantiti yang banyak dalam industry, oleh itu penghasilannya sangat difokuskan. Kajian ini tertumpu terutamanya kepada faktor nutrisi dalam medium fermentasi yang mempengaruhi pertumbuhan dan penghasilan amilase oleh medium fermentasi melalui fermentasi kelompok. Komposisi medium optimum untuk pertumbuhan isolat M dicapai dengan penggunaan teknik satu faktor pada masa. Komposisi medium optimum yang diperoleh ialah sorbitol, ekstrak yis, ammonium nitrat dan kalsium klorida. Seterusnya, kepekatan optimum komposisi medium untuk pertumbuhan dan pengeluaran amilase daripada isolat M telah dicapai dengan aplikasi Reka Bentuk Box-Behnken dan kaedah permukaan tindak balas (RSM). Hasil yang diperoleh untuk kepekatan optimum komposisi medium ialah 5g/L sorbitol, 5g/L ekstrak yis, 2.5g/L ammonium nitrat dan 0.2g/L kalsium klorida. Penghasilan tertinggi amilase ialah 46.31 U/ml aktiviti amilase. Kesimpulannya, dengan pengoptimuman factor ini maka penghasilan amilase akan bertambah. Oleh itu dapat menyumbang dalam industry pembuatan, contohnya detergen.

Kata kunci: Amilase, Faktor Pemakanan, Box-Behnken, RSM

**ABSTRACT**

Amylase is a type of hydrolyte enzyme which is widely used in various applications or industries, including food, brewing, textile, paper, and biofuel industries. Amylase is required in large quantities in industries therefore its production is highly being focused. This study is mainly focusing on nutritional factors in fermentation medium that affect the growth and production of amylase by fermentation medium in laboratory through batch fermentation. The optimum medium composition for the growth of isolate M was achieved by the application of one-factor-at-time technique. The optimum medium composition got are sorbitol, yeast extract, ammonium nitrate and calcium chloride. Next, the optimum concentration of medium composition for the growth and production of amylase from isolate M was achieved by the application of Box-Behnken Design and response surface methodology (RSM). The result get for the optimum concentration of medium composition are 5g/L of sorbitol, 5g/L of yeast extract, 2.5g/L of ammonium nitrate and 0.2g/L of calcium chloride. The highest production of amylase is 46.31 U/ml of amylase activity. In conclusion, by optimizing this factor, amylase production will increase. Therefore, can contribute in the manufacturing industry, for example detergent.

Keywords: Amylase, Nutritional Factors, Box-Behnken, RSM

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Amylases are industrially important enzymes widely used in various applications or industries, including food, brewing, textile, paper, and biofuel industries. Amylase functions to hydrolyze glycosidic bonds in starch into smaller carbohydrate molecules such as glucose and maltose. Amylase can be divided into 3 main classes which are alpha-amylase, beta-amylase, and gamma-amylase. The main source of alpha-amylase is from humans, animals, plants, and microbes, while the main source of beta-amylase is from microbes and plants and lastly the source of gamma-amylase is from animals and plants (Britannica, 2023).

Amylase is a digestive enzyme predominantly secreted by the pancreas and salivary glands and found in other tissues at very small levels (Akinfemiwa et al, 2022). On the other hand, amylase can be produced through microbial fermentation by isolating the amylase producing bacteria in laboratory. The production of these enzymes through microbial fermentation offers a sustainable and cost-effective approach. However, to optimize the production process, it is crucial to understand the factors that influence amylase production by microbial isolates. Nutritional factors play a vital role in regulating enzyme synthesis and secretion in microorganisms, making it an important area of investigation. In short, to grow amylase producing bacteria in laboratory, medium with several nutrients such as carbon source, organic nitrogen source, inorganic nitrogen source and metal ions is needed (Sigma Aldrich, 2023).

Besides, there are numerous studies have been carried out to explore the effect of nutritional factors on amylase production from different microbial isolates. The nutritional factors being tested in previous research include carbon source, nitrogen source, micronutrients or metal ions (magnesium and calcium ions), pH and temperature.

For instance, researchers have investigated the impact of carbon sources on amylase production. Singh et al. (2018) studied the production of amylase from a bacterial isolate and found that the use of starch and maltose as carbon sources has significantly enhanced enzyme production, whereas glucose repressed its synthesis. Similarly, Jain et al. (2020) reported that the utilization of corn starch as a carbon source resulted in higher amylase yields compared to other carbon sources tested (Jain et al., 2020).

Moreover, nitrogen sources have been extensively studied for their influence on amylase production. In a study by Patel et al. (2019), it was observed that the addition of peptone as a nitrogen source stimulated amylase production by a fungal isolate (Patel et al., 2019). Conversely, a study by Li et al. (2017) demonstrated that excess nitrogen concentrations, particularly in the form of ammonium salts, inhibited amylase production in a bacterial strain. This shows that nitrogen sources should only be applied in a suitable amount and should not exceed the limit (Li et al., 2017).

Furthermore, micronutrients and trace elements are also investigated for their roles in amylase production. A study by Khan et al. (2021) demonstrated that the addition of magnesium and calcium ions to the growth medium has significantly enhanced amylase production from a bacterial strain (Khan et al., 2021). Similarly, Oumer et al. (2018) found that the supplementation of zinc and copper improved amylase production by a fungal isolate (Oumer et al., 2018).

The other external factors that affect amylase production include pH and temperature. The pH of the growth medium has also been recognized as a critical factor affecting amylase production. In an investigation by Kumar et al. (2016), it was found that maintaining a slightly acidic pH range of 5.5-6.0 favoured amylase production by a bacterial isolate (Kumar et al., 2016). In contrast, a study by Gupta et al. (2018) revealed that alkaline pH conditions (pH 8.0) were optimal for amylase production from a fungal isolate. Meanwhile, temperature is another vital parameter influencing amylase production (Gupta et al., 2018). Another report reported that a thermophilic bacterial strain exhibited maximum amylase production at 50°C, whereas a mesophilic isolate showed optimal production at 37°C (Al-Abdullah et al., 2019). These findings highlight the importance of temperature optimization based on the microbial isolate.

Overall, the existing research provides us with many valuable insights into the influence of nutritional factors on amylase production from microbial isolates. However,

further investigations are needed to explore additional isolates and optimize the nutritional conditions to maximize amylase yields. This study aims to contribute to this body of knowledge by investigating the effect of specific nutritional factors, including carbon and nitrogen sources, pH, temperature, and micronutrients, on amylase production from isolate M. The findings will aid in developing optimized fermentation processes for enhanced amylase production, thereby benefiting various industries reliant on this valuable enzyme.

## 1.2 Problem Statement

Amylase is an important enzyme that is needed in large quantities for a variety of industrial and biotechnological applications (de Souza & de Oliveira Magalhães, 2010). Nowadays, the usage of amylase is currently increasing because of its usage in starch liquefaction, brewing, sizing of textile industries, paper and detergent industries. Besides that, usage of amylase has also expanded to medical, clinical fields and analytical chemistry (Abd-Elhalim et al., 2023). The demand for amylase is expected to continue to grow as these industries expand and new applications are developed. However, the production rate of amylase naturally from microorganism is low, which leads to the insufficient enzyme production to fulfil the demands. This optimization of amylase production processes may be hindered by the limited understanding of the specific nutritional factors that influence the synthesis and secretion of these enzymes. Therefore, it is important to investigate the effect of nutritional factors on amylase production from microbial isolates to develop optimized fermentation processes that maximize enzyme yields. For example, the maximum yield of α-amylase is 15.15 U/ml from *Bacillus sp.* NRC22017 and the production rate increases after optimisation (Elmansy et al., 2018).

Since many previous studies have explored the impact of nutritional factors on amylase production, the specific nutritional requirements for different microbial isolates should be more comprehensive understood. Understanding the nutritional requirements for amylase production is crucial for achieving high yields and cost-effective production processes. The optimized fermentation conditions can enhance amylase production, leading to improved process efficiency and reduced production costs. Moreover, by

identifying the specific nutritional factors that stimulate or inhibit amylase production, it allows the researchers to tailor the growth medium and optimize the nutritional parameters for each microbial isolate in order to maximize enzyme yields.

There are two methods to modify and increase the production of amylase which are genetic engineering and optimisation of media component (Elyasi Far et al., 2020). This study is mainly focusing on the optimization of media components to increase the production of amylase from isolate M. The findings from this research contribute to the development of optimized fermentation processes for enhanced amylase production, enabling industries to meet the growing demand for these valuable enzymes.

## 1.3 Objectives

The objectives of this study are:

i. To screen carbon source, nitrogen source and metal ions composition for the amylase production from Isolate M.
ii. To apply the best carbon source, nitrogen source and metal ions for optimisation of amylase production from Isolate M using Design Expert Software.

## 1.4 Scope of Study

In this study, the nutritional factors that affect the growth of amylase were investigated. Several carbon sources, organic nitrogen sources, inorganic nitrogen sources and metal ions were added in several growth medium to test the effectiveness in growing amylase. The main source which is the amylase producing bacteria was obtained from stock culture in the previous study (Lim, 2022). Before optimizing the growth medium, the selected amylase-producing bacteria was used to prepare an inoculum. The inoculum will ensure the presence of a sufficient number of viable cells for subsequent fermentation experiments.

During the optimization of the growth medium, the experimental design techniques, such as Response Surface Methodology (RSM), was employed to optimize

the combination of several nutrients in the growth medium. RSM allowed for the systematic variation of factors and generates response data, which can be analyzed to determine the optimal levels of the nutritional factors for maximum amylase production. Once optimization has completed, the study was identified and recorded the optimum combination of carbon sources, organic nitrogen sources, inorganic nitrogen sources, and metal ions in the growth medium. This information was served as a reference for subsequent amylase production experiments.

It is important to note that this study focuses primarily on the nutritional aspects of amylase production and optimization. Other factors, such as pH, temperature, and fermentation conditions, may influence amylase production but are not within the scope of this particular study. The study aims to provide insights into the nutritional requirements for efficient amylase production and contribute to the development of optimized growth media. The results obtained will contribute to the understanding of amylase production and support industries that utilize amylase in various applications.

## 1.5 Significances of Study

The significance of this study lies in its potential contributions and benefits in the field of amylase production and its application in various industries. The study provides valuable insights into the nutritional factors or conditions that affect the large-scale production of amylase. By investigating the effects of different carbon sources, organic and inorganic nitrogen sources, and metal ions, the study helps identify the optimal growth conditions necessary for large-scale production of amylase. This knowledge assists in designing optimized fermentation processes and improving the efficiency of amylase production and enable industries to develop cost-effective and sustainable production processes, leading to increased yields and improved profitability.

As for environmental benefits, the large-scale amylase production can help reduce the reliance on harsh chemicals in industrial processes. Additionally, amylase can be used to break down starch-based waste products, leading to a reduction in environmental pollution. By providing insights into the nutrient requirements for amylase production,

this study supports the development of environmentally friendly and sustainable processes.

By understanding the nutritional factors that affect amylase production, it plays crucial role for the commercialization of amylase-based products. With optimized production processes, the cost-effectiveness and feasibility of amylase-based products can be improved, opening doors for wider market adoption and commercial success.

Besides, this study also contributes to the broader field of biotechnology by expanding our knowledge of enzyme production processes. By focusing on amylase in this experiment, it helps increase our understanding of the factors influencing microbial enzyme synthesis and secretion. This knowledge can be applied to other enzyme production processes, promoting advancements in biotechnological applications.

In summary, this study's significance lies in its potential to improve the efficiency and sustainability of amylase production, leading to a range of benefits for industries and the environment. By investigating the nutritional factors affecting amylase production, the study provides valuable insights that can be applied to optimize production processes, meet industry demands, and promote environmentally friendly practices.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Amylase

Amylase is a digestive enzyme predominantly secreted by the pancreas and salivary glands and found in other tissues at very small levels. Amylase plays an important role in the breakdown of complex carbohydrates to simple sugars. There are two classes of amylases which are endo-amylase and exo-amylase. The classes of amylase are divided according to their mode of action. Endo-amylase breaks α-1,4 glycosidic bonds between adjoining glucose units present in the starch chain, while exo-amylase either catalyse the cleavage of α-1,4 glycosidic bond or catalyse the cleavage of both α-1,4 and α-1,6 glycosidic linkages like glucoamylase and α-glycosidase. Other than classes, amylase can also be classified into three types which are α-amylase, β-amylase and γ-amylase. The type of classification of amylase is according to their cleavage site. Alpha-amylase breaks down the internal α-1,4 glycosidic bonds present in the starch chain while beta-amylase hydrolyses the second α-1,4 glycosidic linkage and gamma-amylase catalyses hydrolysis of α-1,4 and α-1,6 glycosidic bonds. Gamma-amylase acts as the most effective enzyme when the environment is acidic (Joshi et al., 2021).

Amylase is widely used in various industries, including food, pharmaceuticals, and biofuels. The production of amylase can be achieved through microbial fermentation, and bacterial isolates are known to produce high amounts of amylase. To increase the production of enzyme, two methods can be use which are genetic engineering and optimization of medium. There are redundancies in genetic code that amino acid might be encoded by multiple synonymous codons. This scenario gives an opportunity to choose a codon other than the naturally occurring one in the genome to optimize the production with heterologous expression system (Anbu et al., 2017). On the other hand, the optimisation method can be physical factors and nutritional factors. The

common physical factors are temperature and pH. On the other hand, the common nutritional factors will be the medium composition such as carbon source and nitrogen source (Khusro et al., 2017).

Several studies have investigated the effect of nutritional factors on amylase production. For example, some researchers found that the concentration of carbon and nitrogen sources significantly impacted the growth and amylase production of *Bacillus amyloliquefaciens* (Abd-Elhalem et al., 2015). Furthermore, there has been research on the use of statistical tools, such as response surface methodology (RSM), for optimizing the growth conditions for amylase-producing microorganisms (Sharif et al., 2023).

## 2.2 Nutritional Factors Affecting the Production of Amylase

### 2.2.1 Carbon Source

The carbon source is an important factor in the growth of amylase-producing bacteria and any living cell. Carbohydrates which are a type of carbon source is the primary carbon source for the growth of amylase-producing microorganisms. Glucose, sucrose, maltose, and starch are commonly used carbon sources for the production of amylase. Carbon sources will provide energy for the growth of amylase producing bacteria. According to several studies, the type of carbon sources significantly affects the production of amylase (Singh et al., 2018). The commonly used carbon sources are glucose, maltose, starch and sorbitol.

Several studies shows that different amylase producing bacteria needs different carbon source as nutrient. A study shows that *Aspergillus oryzae* S2 needs starch as carbon source to produce alpha-amylase. From another study, molasses act as the carbon source to produce alpha-amylase from *Penicillium notatum* IBGE 03. On the other hand, *Bacillus amyloliquefaciens* needs glucose as the carbon source is shown in another study (Elyasi Far et al., 2020).

Most heterotrophs and chemoheterotrophs need organic compound to serve as an energy source. How fast an organism can metabolise a particular carbon source often determine how fast it can grow (Elbeshbishy, 2014).

### 2.2.2 Nitrogen Source

Nitrogen makes up 14% of the dry weight of most organisms including microorganisms. It is a combination of proteins, nucleic acids and certain essential metabolites, so all living things require a source of nitrogen (Boyd, 2020).

Nitrogen is an essential nutrient for the growth of microorganisms. It can be divided into two which are inorganic nitrogen source and organic nitrogen source. Both sources can be used for the production of amylase. The type of nitrogen sources can significantly affect the production of amylase (Sivaramakrishnan et al., 2006).

### 2.2.2 (a) Organic Nitrogen Source

An organic nitrogen source is a compound that contains nitrogen in its organic form. Organic nitrogen sources are commonly used by microorganisms as a source of nitrogen for growth and metabolic processes. Organic nitrogen is more preferable compared to inorganic nitrogen is because organic nitrogen provides a more diverse range of nitrogen-containing compounds that can be used for biosynthesis (Kumar & Yadav, 2014). The commonly used organic nitrogen sources are peptone, beef extract and urea.

Organic nitrogen source is often used to promote the growth of microorganisms and enhance enzyme production. It contains a complex mixture of organic nitrogen compounds, including amino acids, peptides, nucleotides, vitamins, and minerals. The amino acids and peptides can serve as a source of nitrogen and carbon for the growth of microorganisms. The presence of these compounds can also stimulate the expression of genes involved in the biosynthesis of enzymes such as amylase. Beside amino acids and peptides, it also contains other nutrients like vitamins and minerals that can promote the growth of microorganisms and enhance enzyme production (Pandey et al., 2000).

### 2.2.2 (b) Inorganic Nitrogen Source

Inorganic nitrogen sources are a type of nitrogen compound that does not contain any carbon-hydrogen bonds. These sources are commonly used in microbiology and biotechnology for the growth and biosynthesis of microorganisms and enzymes. Inorganic nitrogen sources are generally less complex than organic nitrogen sources like peptone and beef extract, but they can provide a simpler and more defined growth medium for the growth and biosynthesis of microorganisms and enzymes such as amylase (Madigan et al., 2018). The commonly used inorganic nitrogen sources for the growth of amylase are ammonium sulphate, ammonium nitrate and sodium nitrate.

Inorganic nitrogen provides a readily available source of nitrogen for microorganisms. It can be rapidly assimilated into the cells of isolate M and used for biosynthesis. This allows the microorganism to focus its energy on producing enzymes such as amylase rather than synthesizing nitrogenous compounds. Besides that, it can also provide a source of nitrate ions, which can be used by some microorganisms as a terminal electron acceptor in respiration. This can promote the growth of microorganisms under anaerobic conditions and enhance the production of amylase (Kumar & Pandey, 2013).

### 2.2.3 Metal Ions

The roles for metals in metal activated enzymes and metalloenzymes is to act as electrophilic catalysts, stabilizing the increased electron density or negative charge that can develop during reactions. Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ was reported to increase a-amylase activity of an alkaliphilic *Bacillus sp.* ANT-6 (Pandey et al., 2000). Since $Ca^{2+}$ ions play an important role in alpha-amylase production, therefore calcium chloride ($CaCl_2$) is always the ingredient added in the culture media to produce alpha-amylase. The quantity of the $Ca^{2+}$ ions in calcium chloride should be handled carefully and make sure added in the suitable range to optimize the growth of the amylase.

## 2.3 Application of Amylase

Amylase needs to be produced in large amount is because amylases have latent application in a large number of industrial processes such as food, detergent, textile and pharmaceutical industries (Abd-Elhalim et al., 2023).

In food industry, especially processed food industry like baking, brewing and fruit juice industry needs amylase in a large quantity. For example, amylase is used in bakery industry for the purpose of degrading starch in the flour into smaller dextrin and then fermented by the yeast by adding it into dough (Raveendran et al., 2018).

Besides food industry amylase is also needed in detergent industry. Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergent's ability to remove tough stains and making the detergent environmentally safe. By adding amylase in detergents, stains of starchy foods such as potatoes, gravies, custard and chocolate can be easily removed (Niyonzima & More, 2014).

On the other hand, textile industry also consume amylase. The main uses of amylases in textile industry are resizing. Starch acts as a sizing agent will confirm a fast and secure weaving process. Starch is being preferred as its economy price and is easily to get. Resizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. The α-amylases remove selectively the size and do not attack the fibre (Montazer & Harifi, 2018).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

The materials and apparatus used in the research are listed out as depicted in Table 3.1.

**Table 3.1**: List of materials and apparatus.

| Materials | Apparatus |
|---|---|
| Isolate M from soil in UMK | Petri dish |
| Starch agar powder | Autoclave machine |
| Distilled water | Bunsen burner |
| Gram's iodine | Incubator |
| Soluble starch | Chiller |
| Yeast extract | Inoculating needle |
| Magnesium sulphate | Water Bath |
| Monopotassium phosphate | Conical flask 250 mL |
| Calcium chloride | Beaker 100, 500 and 1000 mL |
| Glucose | Electronic balance |
| Maltose | Shaking incubator |
| Sorbitol | Centrifuge tube 2mL |
| Peptone | Centrifuge machine |
| Magnesium chloride | Pipette |
| Beef extract | Pipette tip |
| Urea | Cuvette |
| Ammonium sulphate | UV-VIS Spectrophotometer |
| Ammonium nitrate | Vortex |
| Sodium nitrate | Test tube 10ml |
| Magnesium sulphate | |
| Zinc sulphate | |
| 3,5-dinitrosalicyclic acid | |
| Sodium Hydroxide | |

## 3.2 Methods

### 3.2.1 Source of microbes

Isolate M was obtained from local stock culture of the previous study from Lim (2022). The stock culture was stored in the freezer of Microbial Technology Lab, UMK.

### 3.2.2 Preparation of starch agar plate

Starch agar (3% w/v) plate was prepared by adding 15 g of starch agar powder with 500ml distilled water. The mixture of starch agar powder and distilled water was stirred welly and was poured into bottles. The bottles with the agar mixture were then be autoclaved for 15 minutes. After that, the bottles were cooled down until it reached 55 °C. Subsequently, the agar mixture was poured into several sterile petri dish. After drying, petri dishes were enclosed with parafilm and were stored upside down with the plastic sleeve in chiller at 4 °C.

### 3.2.3 Screening and selecting potent amylase producing bacteria using starch hydrolysis test

Stock culture from sample was streaked on starch agar plate with the aid of sterile inoculating needle. The starch agar plates as a master plate were then incubated for 24 - 48 hours at 37 °C. Subsequently, single colony was streaked again on another starch agar plate and incubated for 24 - 48 hours at 37 °C for the use of starch hydrolysis test. After 24 hours incubation, Gram's iodine was floated into the plates for several seconds to observe clear zone for detection of amylase producing bacteria.

### 3.2.4 Inoculum Preparation

The amylase producing bacteria that have been observed at clear zone was streaked again on another agar plate from the master plate. The plate was incubated for 24 hours in incubator at 37 °C. After incubation, a loop of colony from that plate was inoculated in test tubes with nutrient broth. The test tubes were incubated for 3 - 4 hours in incubator at 37 °C until the optical density (OD) measurement reached 0.5 by using UV-VIS spectrophotometer at 540 nm.

### 3.2.5 Fermentation

#### 3.2.5 (a) Optimization of production medium

The inoculum 2.5 % (w/v) was inoculated into a 250 mL conical flask containing 50 mL fermentation medium. The fermentation medium is composites of 5 g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L $KH_2PO_4$, and 0.25 g/L $CaCl_2 \cdot 2H_2O$ (Abdel-Fattah et al., 2013; Lim, 2022). The medium composition was set as the control growth medium. The conical flask was incubated for 48 hours in shaking incubator at 37°C and 150 rpm. This step was repeated by substituting the medium composition with substitution medium component as shown in Table 3.2.

**Table 3.2**: Summary of all medium components and the substitution components

| Medium components (source) | Composition percent (g/L) | Control growth medium | Substitution |
|---|---|---|---|
| **Carbon** | 5.0 | Starch | Glucose |
| | | | Maltose |
| | | | Sorbitol |
| **Organic Nitrogen** | 5.0 | Peptone | Beef extract |
| | | | Urea |
| **Inorganic Nitrogen** | 2.5 | Ammonium sulphate | Ammonium nitrate |
| | | | Sodium nitrate |
| **Metal Ions** | 0.2 | $CaCl_2$ | $MgCl_2$ |
| | | | $MgSO_4$ |
| | | | $ZnSO_4$ |

The best medium components get were continue being tested to get the optimum concentration value using Box-Behnken design. Total of 4 variables which are the best carbon source, the best organic nitrogen source, the best inorganic nitrogen source and the best metal ion were included for selection. A centre point (0), lower level (-1) and higher level (+1) were added for the variables as shown in Table 3.3. The fermentation process was same as 3.2.4(a).

**Table 3.3**: Summary of all medium components and the substitution components

| Variables | Actual variable at coded variable levels (g/l) | | |
|---|---|---|---|
| | **Lower (-1)** | **Centre (0)** | **Higher (+1)** |
| **The best carbon source** | 2.0 | 5.0 | 8.0 |
| **The best organic nitrogen source** | 2.0 | 5.0 | 8.0 |
| **The best inorganic nitrogen source** | 1.5 | 2.5 | 3.5 |
| **The best metal ion** | 0.1 | 0.2 | 0.3 |

### 3.2.5 (b) Extraction of the crude enzyme from culture media

The culture fluid from section 3.2.4 (a) was collected after incubation. The culture fluid was centrifuged at 6000 rpm for 10 minutes to get the supernatant as a crude enzyme. The crude enzyme was proceeded to DNS assay. Before DNS assay, the crude enzyme was stored in freezer at 4 °C for future use.

### 3.2.6 Determination of amylase activity using DNS assay

### 3.2.6 (a) Enzyme Assay

DNS method was used to determine the amylase activity. 1 % starch solution was dissolved in sodium hydroxide with the pH of 7. 1 ml of crude enzyme as in section 3.2.4 (b) was mixed with 1ml of starch solution in a test tube. The mixture in test tube was then be incubated in

water bath for 30 minutes in 37 °C. Then, 2 ml of DNS solution was added into the mixture in test tube to stop reaction. Then, let the mixture of crude enzyme, starch solution and DNS solution stay still in room temperature for 10 minutes. Then, the mixture of crude enzyme, starch solution and DNS solution was boiled in water bath for 10 minutes in 100 °C. Then, the mixture of crude enzyme, starch solution and DNS solution was cooled down under tape water. The colour intensity was observed using UV-VIS spectrophotometer at 540 nm. A blank sample with distilled water was used for comparison. The colour intensity was observed three times to get the average value.

### 3.2.6 (b) Formation of glucose standard curve

Glucose standard curve was plotted. Glucose standard curve was used to determine the amount of reducing sugar produced by the amylase enzyme from isolate M. 7 test tubes were prepared and different concentrations of glucose as listed in Table 3.4 was added into it. Then, 2ml of DNS was added into each test tubes. Each test tubes were boiled at 100 °C for 10 minutes in a water bath. The absorbance value of each concentration of glucose was measured by using spectrophotometer at 540 nm. A graph was plotted. The x-axis of the graph was the concentration of glucose (mg/ml) while y-axis was the absorbance reading at 540 nm.

### 3.2.6 (c) Amylase activity

Based on equation 3.2, the y value represents the absorbance value at 540nm while the x value represents the concentration of glucose. The absorbance value got from section 3.2.5 (a) was inserted into y of the equation 3.1. Equation 3.1 was from the glucose standard curve from section 3.2.5 (b) to calculate the x value. After that, the x value was inserted to equation 3.2 to calculate the amylase activity (U/ml).

$$y = 0.0025x$$

Equation 3.1

$$Amylase\ activity\ (U/ml) = \frac{Glucose\ concentration\ from\ spectrophotometer\ \times 1000\ \times dilution\ factor}{Molecular\ weight\ of\ glucose\ \times Incubation\ hour\ (\text{min}) \times Volume\ of\ enzyme\ added}$$

Equation 3.2

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Starch Hydrolysis Test

The isolate M was obtained from the local stock culture in Microbial Technology Lab, UMK. The isolate was grown on 3% w/v starch agar plate. The extracellular amylase enzyme of bacteria is capable of hydrolysing starch into maltose and glucose. Conversely, not every bacterium possesses the ability to generate amylase enzymes.

A biochemical test known as the starch hydrolysis test determines whether or not bacteria (microorganisms) are capable of producing amylase and utilizing starch as a carbon source. It is frequently employed to distinguish *Bacillus, Clostridium, Fusobacterium, Streptococcus, Enterococcus, and Pseudomonas species* (Aryal, 2018). Iodine was added to the agar in order to enable the interpretation of the results of the starch hydrolysis test. A dark brown color was produced as a result of the reaction between the iodine and the starch. The amylose chain's spiral conformation, facilitated by the α-linkages, contributes to the formation of a soluble dark blue starch-iodine complex with iodine reagent. This complex acquires its blue hue when triiodide ions from the iodine reagent enter the spiral structure (Lal & Cheeptham, 2012; Tetlow & Bertoft, 2020). Consequently, the hydrolysis of the starch will result in the formation of a clear zone surrounding the bacterial growth of M as shown in Figure 4.1(B). When α-linkages are absent, such as *Shewanella oneidensis* (MR-1), no clear zone formed as shown in Figure 4.2(B). Figure 4.2 shows the comparison of the hydrolysis test between isolate M with the presence of α-linkages and *Shewanella oneidensis* (MR-1) without the presence of the α-linkages.


**Figure 4.1:** Before (A) and after (B) hydrolysis test of Isolate M

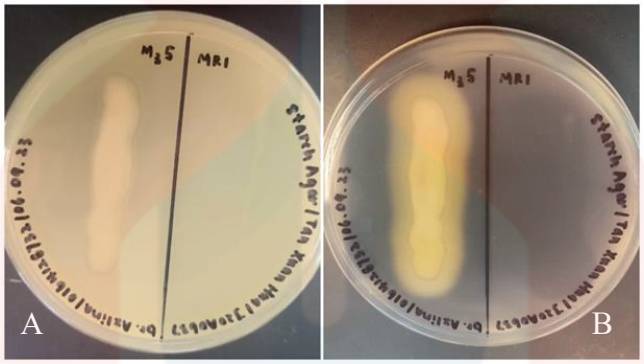


**Figure 4.2:**

Comparison of hydrolysis test between isolate M and *Shewanella oneidensis* (MR-1)

4.2 (A) Before hydrolysis test, 4.2 (B) After hydrolysis test

## 4.2 Identification of suitable growth medium components for amylase

The enzyme production was found to be substantially influenced by carbon, inorganic nitrogen, organic nitrogen and metal ions, as determined by the one factor at a time (OFAT) method. Following this, the amylase activity of each source was calculated by using the equation as stated in section 3.2.5 (c).

### 4.2.1 Effect of carbon source on amylase production

Carbon sources play a crucial role in the production of amylase, an enzyme that catalyze the breakdown of starch into simpler sugars. Different carbon sources can have variable effects on the amylase production. To screen the most suitable carbon source for the production of amylase, different carbon source such as starch, glucose, maltose and sorbitol were replaced at the same concentration (5 g/L) in the fermentation medium. According to Figure 4.3, different carbon source has significantly influenced the production of amylase. Sorbitol gets to produce maximum amount of amylase activity (72.61 U/ml). Meanwhile starch showed lowest production of amylase during 24 hours fermentation.

According to research of Sudharhsan et al (2017), a high enzyme yield (31 U/ml) was observed in lactose-based production media using the isolated strain. Amylase production was inhibited by glucose and fructose, respectively. In a similar vein, Teodoro and Martins (2000) have documented that the production of carbohydrate degrading enzyme by the majority of *Bacillus sp.* results in catabolic inhibition facilitated by easily metabolized substrates like glucose and fructose. Amylase production is inhibited in the hyperthermophilic archeon *Sulfolobus solfataricus*, according to findings reported by Haseltine et al. (1996). The findings where a bit differ from this research. It might be the strain of bacteria is different so the carbon source needed for growth was also different.

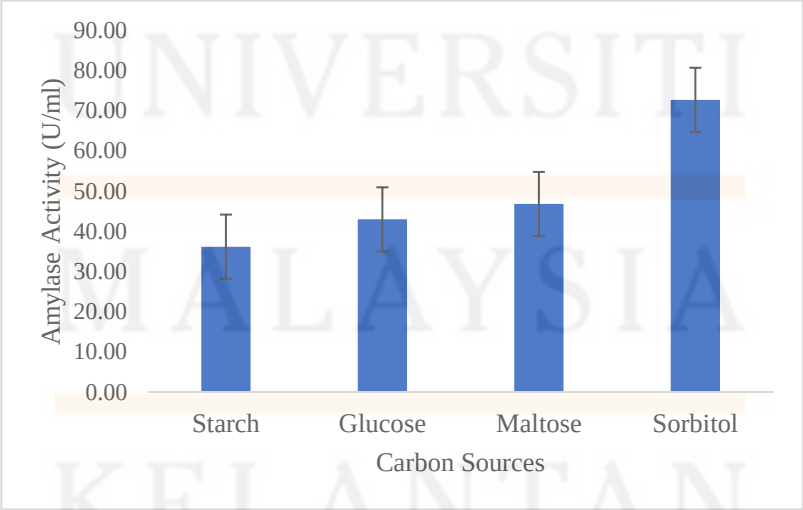


**Figure 4.3:** Effect of carbon source on amylase production from Isolate M

### 4.2.2 Effect of organic nitrogen source on amylase production

The nature and concentration of carbon and nitrogen source in the culture medium are reported to important factors governing bacterial growth and amylase production (Akeel and Umar, 2010; Lal et al., 2016). In this study, inorganic nitrogen source was eliminated from the fermentation medium to identify the effect of sole organic nitrogen source on amylase production. To screen the most suitable organic nitrogen source for the production of amylase, different organic nitrogen source such as yeast extract, peptone, beef extract and urea were replaced at the same concentration (5 g/L) in the fermentation medium. According to Figure 4.4, different organic nitrogen source has significantly influenced the production of amylase. Yeast extract gets to produce maximum amount of amylase activity (71.50 U/ml).

The result of Shoukry's research was similar with this research as the results showed that yeast extract was the best organic nitrogen source that its amylase activity reached 0.340 U/ml (Shoukry et al., 2017).

Shaista et al. (2003) founded that maximum amylase activity upon supplement with 0.2% peptone as an inorganic nitrogen source for *Bacillus sp.* (Shoukry et al., 2017, Shasita et al., 2003).

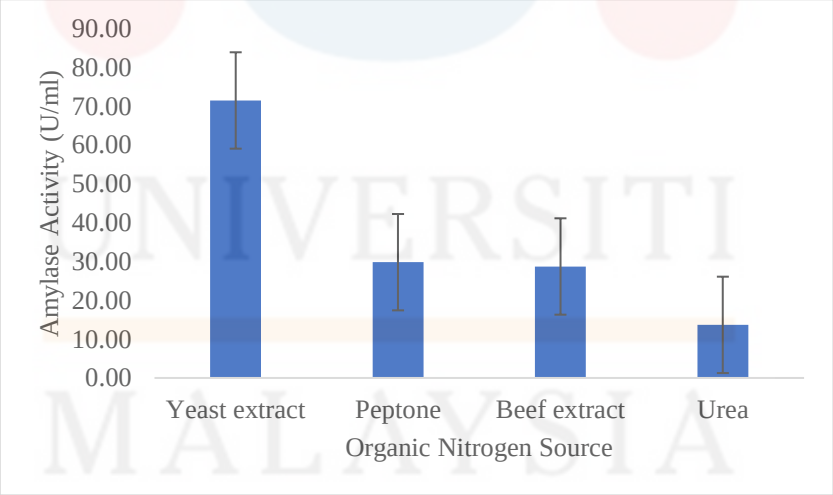


**Figure 4.4:** Effect of organic nitrogen source on amylase production from Isolate M

### 4.2.3 Effect of inorganic nitrogen source on amylase production

The nitrogen sources are of secondary energy source to organisms, which play an important role in the growth and enzyme production. In this study, organic nitrogen source was eliminated from the fermentation medium to identify the effect of sole inorganic nitrogen source on amylase production. To screen the most suitable organic nitrogen source for the production of amylase, different inorganic nitrogen source such as ammonium sulphate, ammonium nitrate and sodium nitrate were replaced at the same concentration (2.5 g/L) in the fermentation medium. According to Figure 4.5, different inorganic nitrogen source has significantly influenced the production of amylase. Ammonium nitrate gets to produce maximum amount of amylase activity (32.42 U/ml). Due to elimination of organic nitrogen source, the amylase activity was decreased. This shows that inorganic nitrogen source does not affect much in amylase production.

The information presented in the research paper from Shatta et al indicates that the type of inorganic nitrogen source had no discernible impact on amylase production. Ammonium nitrate was, on average, marginally more suitable than ammonium sulphate in this context due to its amylolytic activity was the highest which is 3.4 U/ml. This finding is consistent with the results reported with other inorganic nitrogen source (ammonium sulphate and potassium nitrate), which indicated that ammonium nitrate provided the most optimal nitrogen source for amylase production (Shatta et al., 1990).

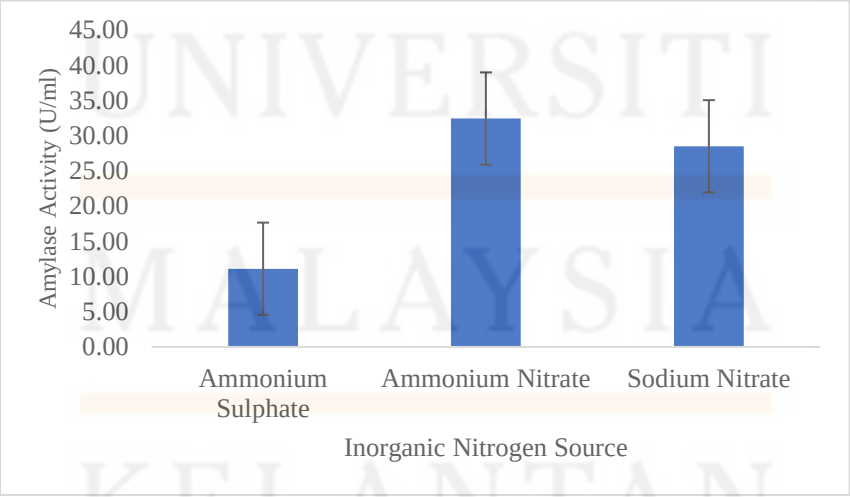


**Figure 4.5:** Effect of inorganic nitrogen source on amylase production from Isolate M

### 4.2.4 Effect of metal ions on amylase production

To screen the most suitable metal ions for the production of amylase, different metal ions such as calcium chloride, magnesium chloride, magnesium sulphate and zinc sulphate were replaced at the same concentration (0.2 g/L) in the fermentation medium. According to Figure 4.6, different metal ions has significantly influenced the production of amylase. Calcium chloride gets to produce maximum amount of amylase activity (70.89 U/ml). Calcium chloride was the metal ion that gives the highest production of amylase producing bacteria is because the $Ca^{2+}$ ions in calcium chloride is present in alpha-amylase structure. Hence it plays an important role in alpha-amylase production (Elyasi Far et al., 2020).

Metal ions act as trace element also require for microbial growth Trace element fulfils a variety of essential function in the cell. Some serves as a cofactor (V. Albergoni & E. Piccinni, 1983). The impact of metal ions at an initial concentration of 0.1% has been investigated. The findings presented illustrate the impact of different metal ions on the amylase productivity of strains of *B. subtilis*. The results obtained indicate that the production of amylase was influenced by the metal ions, with variations observed among the producer strains. An enzyme production increase of significance was observed when $FeCl_3$ and $MgSO_4$ were added to the bacterial fermentation medium of the strains under investigation. The media containing $MgSO_4$ supplemented the *B. subtilis* strain with the highest amylase activity of 0.454 U/ml (Shoukry et al., 2017). The result was a bit different from this research as the strain of bacteria was different. The metal ions needed for growth was also different.

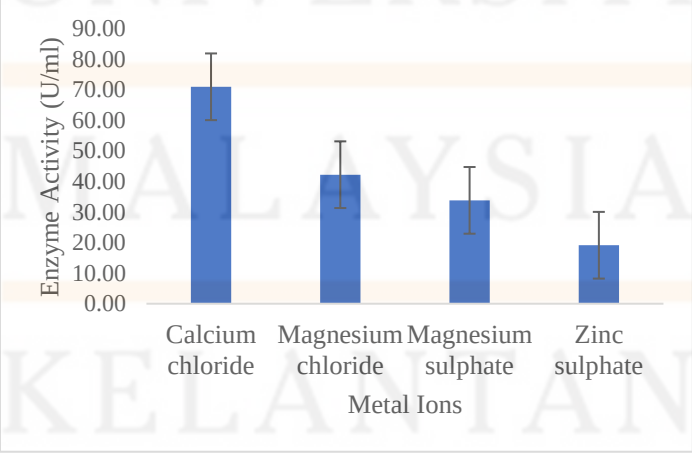


**Figure 4.6:** Effect of metal ions on amylase production from Isolate M

## 4.3 Screening the concentration of production medium

The optimization of amylase production was carried out according to the production medium. To develop the optimum medium composition to produce maximum level of amylase, Box-Behnken Design was used with the aid of Design Expert Version 13 software®.

### 4.3.1 Screening the concentration of medium composition by Box-Behnken design

The response surface methodology (RSM) using Box-Behnken design was applied for medium optimization of amylase production. The Box-Behnken experimental design has been selected to determine the link between the response functions and variables. The Box - Behnken design is a rotatable second-order design that is derived from three-level incomplete factorial designs. The unique configuration of the Box-Behnken design levels enables a proportional increase in the number of design points and polynomial coefficients (Sen et al., 2014). It showed the importance of optimizing medium composition in attaining higher production of amylase. By using Box-Behnken Design, it provides distinct advantages over the optimization approach. It also effectively identified possible interactions between parameters and save time by lowering the number of tests required (Yu & He, 2017). In this study, four variables were tested which are carbon source, organic nitrogen source, inorganic nitrogen source and metal ions. Table 4.1 shows the experimental plan proposed by Box-Behnken design which contained predicted and experimental results. Both of these results showed that the actual response values agreed with the predicted response values. The highest and lowest production was recorded in run order 19 (46.31 U/ml) and 11 (23.93 U/ml) respectively. The highest production is slightly lower than the previous method (OFAT) might because of the inorganic nitrogen factor was not eliminated. In the result of the previous method (OFAT), the production of amylase producing bacteria for inorganic nitrogen source was low (32.42 U/ml) when compared to others were in the range of 70 – 73 U/ml.

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. On the other hand, the equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here,

the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

Coded Equation:

$$\text{Enzyme Activity (U/ml)} = 33.99 + 3.30\,x_1 + 4.12\,x_2 + 3.87\,x_3 + 2.15\,x_4$$

(Equation 4.1)

Actual Equation:

$$\text{Enzyme Activity (U/ml)} = 7.66938 + 1.09889\,x_1 + 1.37333\,x_2 + 3.86500\,x_3 + 21.48333\,x_4$$

(Equation 4.2)

**Table 4.1:** Experimental design of Box-Behnken Design

| Std | Run | $x_1$: Sorbitol (g/L) | $x_2$: Yeast extract (g/L) | $x_3$: Ammonium nitrate (g/L) | $x_4$: Calcium chloride (g/L) | Experimental amylase activity (U/ml) | Predicted amylase activity (U/ml) | Residual value |
|---|---|---|---|---|---|---|---|---|
| **2** | 1 | 8 | 2 | 2.5 | 0.2 | 33.46 | 33.42 | 0.0387 |
| **13** | 2 | 5 | 2 | 1.5 | 0.2 | 39.94 | 41.15 | -1.21 |
| **8** | 3 | 5 | 5 | 3.5 | 0.3 | 36.49 | 40.00 | -3.51 |
| **14** | 4 | 5 | 8 | 1.5 | 0.2 | 43.47 | 40.26 | 3.21 |
| **20** | 5 | 8 | 5 | 3.5 | 0.2 | 31.44 | 34.81 | -3.37 |
| **7** | 6 | 5 | 5 | 1.5 | 0.3 | 25.89 | 33.99 | -8.01 |
| **9** | 7 | 2 | 5 | 2.5 | 0.1 | 28.61 | 35.96 | -7.35 |
| **28** | 8 | 5 | 5 | 2.5 | 0.2 | 36.72 | 39.43 | -2.71 |
| **18** | 9 | 8 | 5 | 1.5 | 0.2 | 36.00 | 34.56 | 1.44 |
| **10** | 10 | 8 | 5 | 2.5 | 0.1 | 33.96 | 32.02 | 1.94 |
| **22** | 11 | 5 | 8 | 2.5 | 0.1 | **23.93** | 26.00 | -2.07 |
| **3** | 12 | 2 | 8 | 2.5 | 0.2 | 37.60 | 34.24 | 3.36 |
| **19** | 13 | 2 | 5 | 3.5 | 0.2 | 33.53 | 33.99 | -0.4597 |
| **15** | 14 | 5 | 2 | 3.5 | 0.2 | 33.19 | 33.99 | -0.7997 |
| **23** | 15 | 5 | 2 | 2.5 | 0.3 | 37.43 | 35.71 | 1.72 |
| **5** | 16 | 5 | 5 | 1.5 | 0.1 | 33.87 | 33.17 | 0.7037 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **1** | 17 | 2 | 2 | 2.5 | 0.2 | 27.49 | 27.98 | -0.4863 |
| **21** | 18 | 5 | 2 | 2.5 | 0.1 | 26.57 | 26.83 | -0.2580 |
| **29** | 19 | 5 | 5 | 2.5 | 0.2 | **46.31** | 41.97 | 4.34 |
| **17** | 20 | 2 | 5 | 1.5 | 0.2 | 30.81 | 28.54 | 2.27 |
| **11** | 21 | 2 | 5 | 2.5 | 0.3 | 33.45 | 33.99 | -0.5397 |
| **6** | 22 | 5 | 5 | 3.5 | 0.1 | 32.68 | 33.73 | -1.05 |
| **12** | 23 | 8 | 5 | 2.5 | 0.3 | 29.83 | 27.72 | 2.11 |
| **4** | 24 | 8 | 8 | 2.5 | 0.2 | 37.48 | 35.14 | 2.34 |
| **24** | 25 | 5 | 8 | 2.5 | 0.3 | 33.37 | 32.84 | 0.5287 |
| **16** | 26 | 5 | 8 | 3.5 | 0.2 | 44.76 | 41.41 | 3.35 |
| **26** | 27 | 5 | 5 | 2.5 | 0.2 | 33.42 | 32.27 | 1.15 |
| **27** | 28 | 5 | 5 | 2.5 | 0.2 | 35.52 | 33.99 | 1.53 |
| **25** | 29 | 5 | 5 | 2.5 | 0.2 | 28.48 | 26.57 | 1.91 |

** The bold values indicated the highest and the lowest amylase activity

*** The runs in the red box are the repetition runs generated by the software

There are five repetitions of runs with the same concentration of four factors. The result should be almost same. But in the result, the enzyme activity was in the range of 28 – 46 U/ml, which considered quite a big gap of range. This might be because the error when weighing the nutrients.

The F-value derived from an ANOVA hypothesis test is used to assess if there is a statistically significant disparity in the means of three or more data groups. The F-value is the ratio of the sum of squares for the difference between each group mean and the grand mean to the sum of squares for the difference between individual values and the group mean (Feldman, 2018).

In this study, the model F-value of 14.06 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. On the other hand, models with p-value less than 0.0500 indicate model terms are significant. In this case $x_1$, $x_2$, $x_3$ and $x_4$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Based on Table 4.2, all models which are sorbitol, yeast extract, ammonium nitrate and calcium chloride have the p-values lower than 0.0500 which means shows significant effect on amylase production.

The Lack of Fit F-value of 0.68 implies the Lack of Fit is not significant relative to the pure error. There is a 75.10% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good.

**Table 4.2:** ANOVA for linear model of Box-Behnken Design

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 568.75 | 4 | 142.19 | 14.06 | < 0.0001 | significant |
| **$x_1$-Sorbitol** | 130.42 | 1 | 130.42 | 12.90 | 0.0015 | |
| **$x_2$-Yeast Extract** | 203.69 | 1 | 203.69 | 20.15 | 0.0002 | |
| **$x_3$-Ammonium Nitrate** | 179.26 | 1 | 179.26 | 17.73 | 0.0003 | |
| **$x_4$-Calcium Chloride** | 55.38 | 1 | 55.38 | 5.48 | 0.0279 | |
| **Residual** | 242.64 | 24 | 10.11 | | | |
| **Lack of Fit** | 187.55 | 20 | 9.38 | 0.6810 | 0.7510 | not significant |
| **Pure Error** | 55.08 | 4 | 13.77 | | | |
| **Cor Total** | 811.39 | 28 | | | | |

### 4.3.2 Effect of sorbitol and yeast extract on amylase production

The surface in Figure 4.7 represents a stationary ridge surface (Myers et al., 2009). When the colour turns darker, the responses increased. When the concentration of ammonium nitrate and calcium chloride are minimised which are 1.5 g/L and 0.1 g/L respectively, the amylase activity decreased. When the concentration of ammonium nitrate and calcium chloride are maximised which are 3.5 g/L and 0.3 g/L respectively, the amylase activity increased. In both conditions, the concentration of sorbitol and yeast extract are maintained at 5.0 g/L. Based on Figure 4.7 (C), when the colour gets darker means the amylase activity is increasing. Referring to the contour plot, the maximum response gets when the concentration of both sorbitol and yeast extract are nearly to 8.0 g/L.

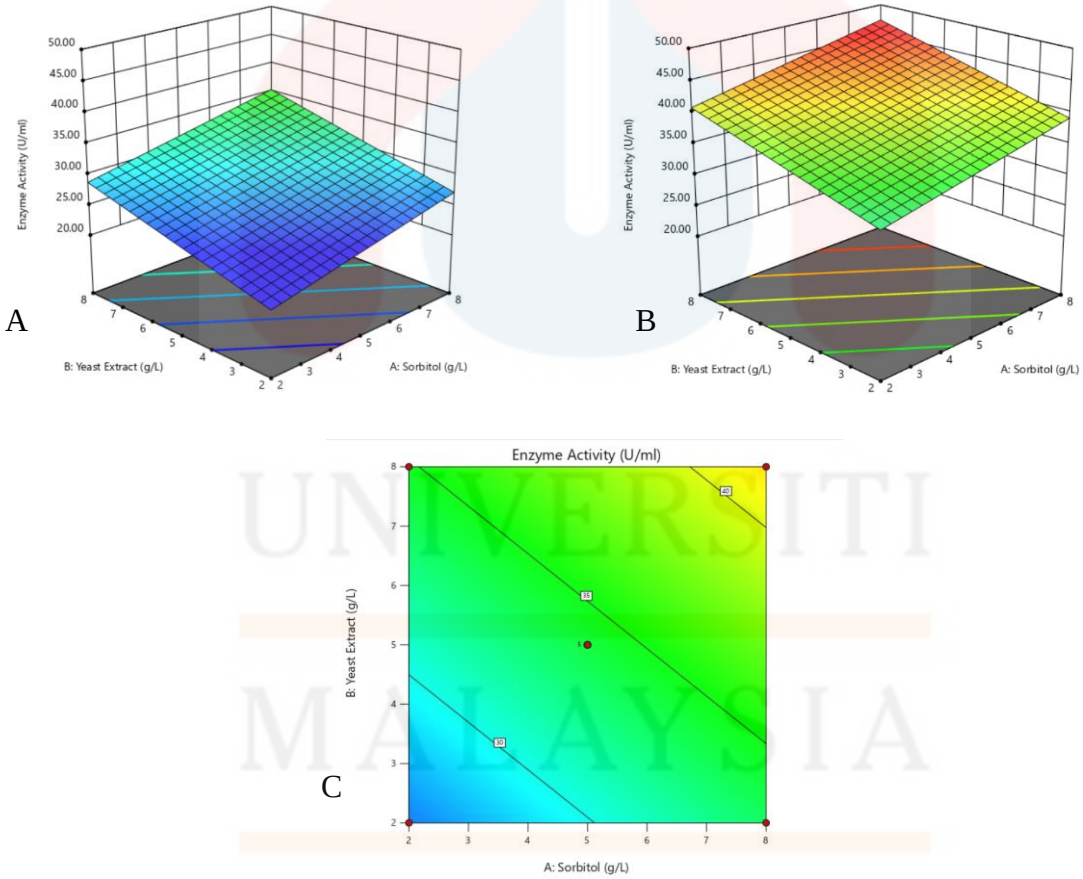


**Figure 4.7:** Surface plot on low concentration (A), high concentration (B) and contour plot (C) of amylase production by Isolate M; the effect of sorbitol and yeast extract concentration

### 4.3.3 Effect of sorbitol and ammonium nitrate on amylase production

The surface in Figure 4.8 represents a stationary ridge surface (Myers et al., 2009). When the colour turns darker, the responses increased. When the concentration of yeast extract and calcium chloride are minimised which are 2.0 g/L and 0.1 g/L respectively, the amylase activity decreased. When the concentration of ammonium nitrate and calcium chloride are maximised which are 8.0 g/L and 0.3 g/L respectively, the amylase activity increased. In both conditions, the concentration of sorbitol and ammonium nitrate are maintained at 5.0 g/L and 2.5 g/L respectively. Based on Figure 4.8 (C), when the colour gets darker means the amylase activity is increasing. Referring to the contour plot, the maximum response gets when the concentration of sorbitol and ammonium nitrate are nearly to 8.0 g/L and 3.5 g/L respectively.

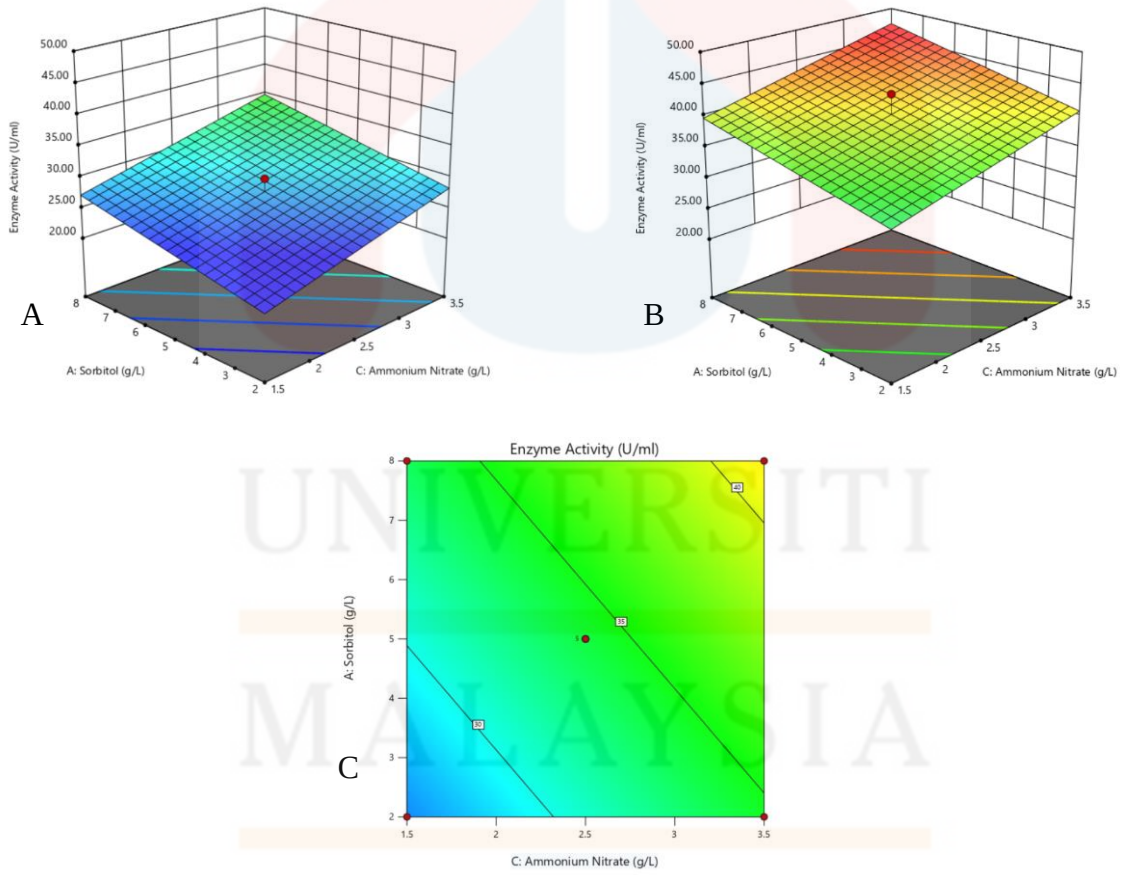


**Figure 4.8:** Surface plot on low concentration (A), high concentration (B) and contour plot (C) of amylase production by Isolate M; the effect of sorbitol and ammonium nitrate concentration

### 4.3.4 Effect of sorbitol and calcium chloride on amylase production

The surface in Figure 4.9 represents a stationary ridge surface (Myers et al., 2009). When the colour turns darker, the responses increased. When the concentration of yeast extract and ammonium nitrate are minimised which are 2.0 g/L and 1.5 g/L respectively, the amylase activity decreased. When the concentration of ammonium nitrate and calcium chloride are maximised which are 8.0 g/L and 3.5 g/L respectively, the amylase activity increased. In both conditions, the concentration of sorbitol and calcium chloride are maintained at 5.0 g/L and 0.2 g/L respectively. Based on Figure 4.8 (C), when the colour gets darker means the amylase activity is increasing. Referring to the contour plot, the maximum response gets when the concentration of sorbitol and calcium chloride are nearly to 8.0 g/L and 0.3 g/L respectively.

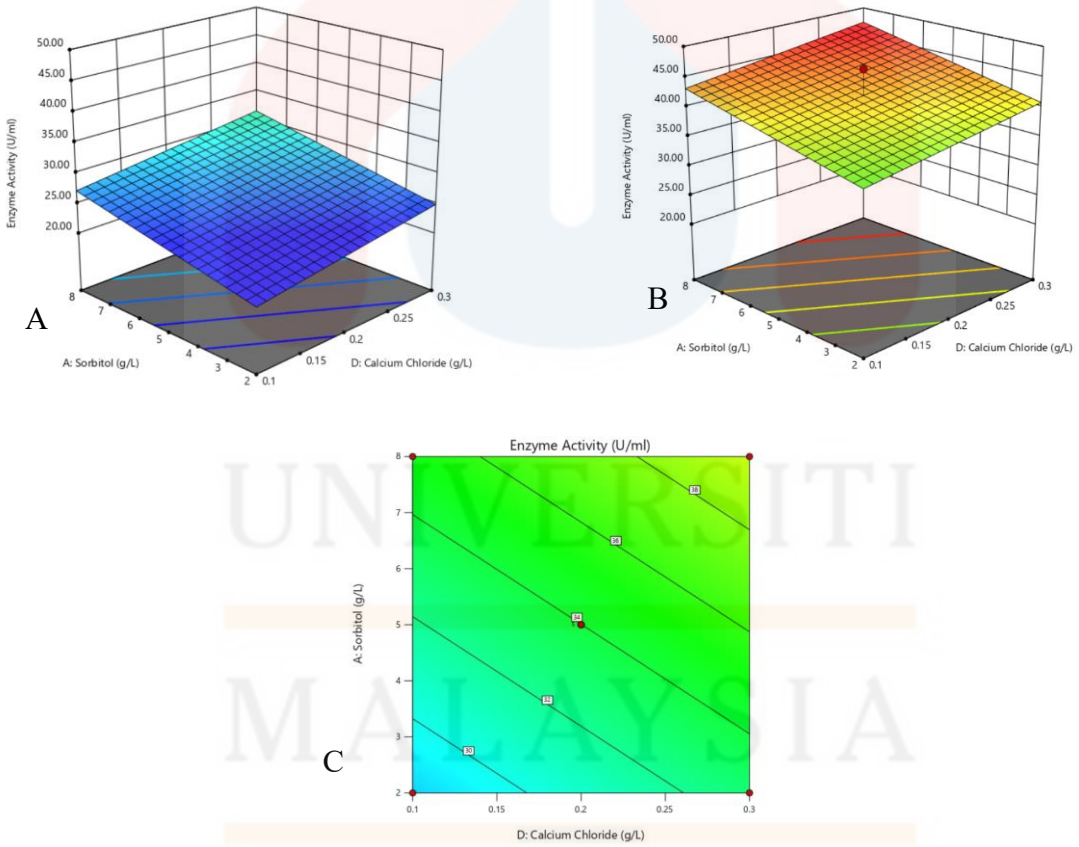


**Figure 4.9:** Surface plot on low concentration (A), high concentration (B) and contour plot (C) of amylase production by Isolate M; the effect of sorbitol and calcium chloride concentration

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

In conclusion, screening and optimising the nutritional factors that affects the amylase production are achieved to produce higher production of amylase from Isolate M.

Based on the first objective of this study, to screen carbon source, nitrogen source and metal ions composition for the amylase production from Isolate M. The best carbon source, organic nitrogen source, inorganic nitrogen source and metal ions were obtained which are sorbitol, yeast extract, ammonium nitrate and calcium chloride respectively. The amylase activity of sorbitol, yeast extract, ammonium nitrate and calcium chloride are 72.61 U/ml, 71.50 U/ml, 32.42 U/ml and 70.89 U/ml respectively.

By using BBD, the second objective of this study was also achieved by getting the best medium composition concentration based on the best sources obtained from first objective. The highest amylase activity obtained is 46.31 U/ml with the medium composition of 5 g/L of sorbitol, 5 g/L of yeast extract, 2.5 g/L of ammonium nitrate and 0.2 g/L of calcium chloride. The lowest amylase activity obtained is 23.93 U/ml with the medium composition of 5 g/L of sorbitol, 8 g/L of yeast extract, 2.5 g/L of ammonium nitrate and 0.1 g/L of calcium chloride.

## 5.2 Recommendations

This study has a wide scope in areas of strain confirmation, screening and optimization. It is recommended that additional studies should be done as proposed below.

This study is recommended to have further improvements in process conditions in physical factors such as temperature, pH, agitation rate and aeration rate. On the other hand, this study focused on nutritional factors which is non-numerical factors, it is more recommended to use RSM to optimize the different source of nutrient by using factorial design. It will get more accurate value to proceed in concentration identification. Furthermore, it is recommended to identify isolate M using molecular identification. Lastly, proper storage condition is recommended to enable the stability of enzyme for long term storage and usage without losing its activity.

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# APPENDIX A

The chemicals listed were used in this project with the abbreviated names of the suppliers.

| Materials | Manufacturers |
|---|---|
| 3,5-dinitrosalicyclic acid | R & M Chemicals, UK |
| Ammonium nitrate | Hmbg Chemicals, Germany |
| Ammonium sulphate | Hmbg Chemicals, Germany |
| Beef extract | R & M Chemicals, UK |
| Calcium chloride | Merck, Germany |
| Glucose | R & M Chemicals, UK |
| Iodine solution | ThermoFisher |
| Magnesium chloride | Merck, Germany |
| Magnesium sulphate | QRec, New Zealand |
| Maltose | R & M Chemicals, UK |
| Monopotassium phosphate | Bendosen, US |
| Nutrient Broth | Oxoid, England |
| Peptone | R & M Chemicals, UK |
| Sodium hydroxide | Hmbg Chemicals, Germany |
| Sodium nitrate | SYSTERM |
| Sodium potassium tartrate | Hmbg Chemicals, Germany |
| Soluble starch | Hmbg Chemicals, Germany |
| Sorbitol | Merck, Germany |
| Starch agar powder | Merck, Germany |
| Urea | Hmbg Chemicals, Germany |
| Yeast Extract | Sigma, USA |
| Zinc sulphate | Hmbg Chemicals, Germany |

**Equipment**

| **Names** | **Manufacturers** |
|---|---|
| Autoclave | Hirayama Mfg, Japan |
| Hot plate and stirrer | Thermoscientific, US |
| Incubator | Jelotech, Korea |
| Microcentrifuge | Hettich, Germany |
| pH meter | Fisher Scientific, UK |
| Shaking incubator | Jelotech, Korea |
| Spectrophotometer | Thermoscientific, US |
| Chiller (4°C) | Puncak steel, Malaysia |
| Freezer (-20°C) | Techlav Mfg. |

| **Computer Software** | **Manufacturers** |
|---|---|
| Design Expert 13 | State-East |
| Microsoft Excel 2007 | United States |

# APPENDIX B

## Preparation of Media, Buffers and Solutions

All medias were autoclaved at 121°C for 15 minutes.

Table B: Preparation of media, buffer and solutions

| Media/Buffers/Solutions | Composition |
|---|---|
| Starch Agar | 15g of starch agar powder, 500ml of distilled water |
| Nutrient Broth | 14g of nutrient broth powder, 500ml of distilled water |
| DNS solution | 1g of 3,5-dinitrosalicyclic acid, 30g of sodium potassium tartrate, 200ml of distilled water |

**APPENDIX C**

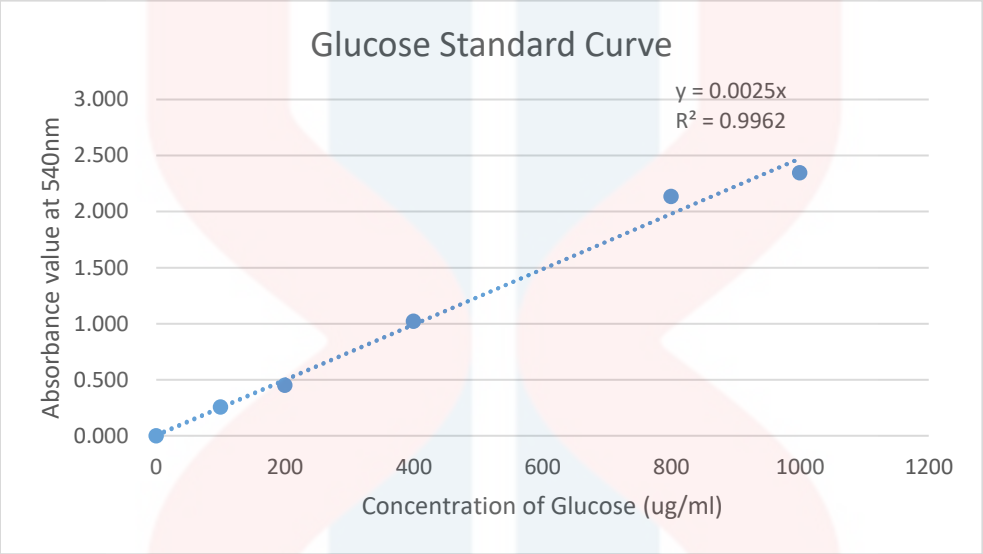


Figure C: Glucose Standard Curve