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EFFECT OF PHYSICAL FACTORS ON THE AMYLASE PRODUCTION FROM ISOLATE M

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DECLARATION

I declare that this thesis entitled "Effect of Physical Factors on Amylase Production from Isolate M" is the result of my own research except as cited in the references.

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KESAN FAKTOR FIZIKAL TERHADAP PENGHASILAN AMILASE DARI ISOLAT M

ABSTRAK

Permintaan untuk enzim amilase telah meningkat di seluruh dunia. Haiwan, tumbuh-tumbuhan dan mikrob adalah beberapa sumber hasil amilase. Dalam pengeluaran enzim amilase, faktor fizikal memainkan peranan penting seperti pH, suhu, pergolakan, masa inkubasi dan saiz inokulum. Dalam kajian ini, faktor fizikal yang disebutkan dinilai dengan nilai yang berbeza. Ia membantu menentukan sama ada faktor fizikal mempunyai keupayaan menghasilkan tinggi atau mempunyai apa-apa kesan ke atas pengeluaran. Terdapat dua objektif untuk kajian ini iaitu mencari masa inkubasi terbaik bgai penghasilan amilase, untuk mengoptimumkan faktor fizikal; pH, suhu, agitasi, masa inkubasi dan saiz inokulum untuk mendapatkan kadar pengeluaran amilase tertinggi dan menggunakan faktor fizikal terbaik; pH, suhu, pergolakan, masa inkubasi dan saiz inokulum untuk mendapatkan pengeluaran amilase tertinggi. Bakteria yang menghasilkan amilase telah disaring menggunakan iodin melalui ujian hidrolisis kanji. Response Surface Methodology (RSM) sebagai alat yang digunakan dalam kajian untuk mengoptimumkan faktor fizikal terbaik yang bekerja pada pengeluaran enzim amilase. Lima pembolehubah bebas yang berbeza dipilih untuk menguji faktor fizikal dalam RSM ini. Selepas menjalankan masa inkubasi yang berbeza, 24 jam adalah masa inkubasi optimum untuk penghasilan amilase tertinggi berbanding dengan 48 jam dan 72 jam. Di antara empat faktor fizikal lain, agitasi dan pH menyumbang kepada pengeluaran lebih banyak pada 150 rpm dan pH 7. Aktiviti amilase tertinggi diperoleh di antara reka bentuk eksperimen 57.65 (U/mL), sementara hasil terendah adalah 2.22 (U/mL). Secara ringkasnya, pengeluaran tertinggi dan terendah diperoleh dari tahap agitasi dan pH yang sama, tetapi kesan itu adalah dari saiz inokulum dan suhu.

Kata kunci: Amylase, faktor fizikal, pengeluaran amilase, RSM, pembolehubah bebas

EFFECT OF PHYSICAL FACTORS ON THE AMYLASE PRODUCTION FROM ISOLATE M

ABSTRACT

The demand for amylase enzymes has been increasing worldwide. Animals, plants and microbials are the several sources of amylase produce. In amylase enzyme production, the physical factors play crucial roles such as pH, temperature, agitation, incubation time and inoculum size. In this study, the physical factors mentioned are evaluated with different values. It aids in determining if the physical factors have the capability of producing high or have any effect on the production. There are two objectives for this study which are to find the best incubation time for the amylase production, to optimize the physical factors; pH, temperature, agitation, incubation time and inoculum size to obtain highest production rate of amylase and to apply the best physical factors; pH, temperature, agitation, incubation time and inoculum size to get highest production of amylase. The amylase producing bacteria were screened using iodine through starch hydrolysis test. Response Surface Methodology (RSM) as tool used in the study to optimize the best physical factors worked on the production of amylase enzyme. Five different independent variables were chosen to test the physical factors in this RSM. After conducting different incubation times, 24 hours was the optimum incubation time for the highest production of amylase compared to 48 hours and 72 hours. Among the other four physical factors, agitation and pH contribute to produce more production at 150 rpm and pH 7. Highest amylase activity was obtained among the experimental design 57.654 (U/mL). In short, the highest and lowest production was obtained from the same level of agitation and pH, but the effect was from inoculum size and temperature.

Keywords: Amylase, physical factors, production of amylase, RSM, independent variables

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SMF	Submerged fermentation	1
SSF	Solid-state fermentation	1
DNS	Dinitrosalicyclic Acid	4
RSM	Response Surface Methodology	3
rpm	Revolutions per minute	11
MgSO4.7H2O	Magnesium sulphate heptahydrate	16
(NH4)2SO4	Ammonium sulphate	16
KH2PO4	Monopotassium Phosphate	16
CaCl2.2H2O	Calcium Chloride Dihydrate	16
BBD	Box-Behnken Design	3
OD	Optical density	21

LIST OF SYMBOLS

α	Alpha	5
β	Beta	5
γ	Gamma	5
℃	degree Celsius	17
v/v	Volume per volume	17

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are essential to our body for digestion, liver function and aid in speeding up the chemical reaction of our body (Robinson, 2015). The changes in temperature and pH level make different enzymes work in varies environment or surroundings. An enzyme is a globular protein that occurs naturally in living organisms, also known as biocatalysts. Enzymes are classified based on their function. The main classes of enzymes include oxidoreductase, transferase, hydrolase, lyases, isomerases, and ligases (de Souza Vandenberghe et al., 2020). Various enzymes are used in most industries as well as on a commercial basis. Amylase is one of the most widely used enzymes in industry. Amylase is an enzyme, a type of protein that helps your body break down carbohydrates. Amylases are classified as alpha, beta, and gamma, respectively. Not only amylase, more than 5,000 enzymes have been discovered in living organisms. All these enzymes are glycoside hydrolases and act on α-1,4- glycosidic bonds (Kherouf et al., 2021).

For instance, α-amylase found in humans, animals, plants and well in some microbe (Kherouf et al., 2021). The pancreas and salivary glands in your mouth make α-amylase. Compared to β- amylase, α-amylase tend to work more quickly. The optimum pH of α-amylase is between 6.7 -7.0 and it categorized under glycosidic hydrolase family 13. The second type, β-amylase is usually found in plants and microbes. It belongs to glycosidic hydrolase family 14 and optimum pH is 4.0-5.0. Lastly, γ-amylase is found in plants and animals. Their optimum pH is 3 (Sundarram & Murthy, 2014). They are a member of the glycosidic hydrolase family 15 (Joshi et al., 2021).

Submerged fermentation (SmF) and solid-state fermentation (SSF) have been utilized in industrial sites for enzyme production purposes. A type of industrial enzyme called amylases makes up about 25% of the market for enzymes, which is used in various industrial processes like those in the sugar, textile, paper, brewing, distilling, and pharmaceutical industries. Submerged fermentation (SmF) has been historically used for assembly of industrially necessary

enzymes because of the benefit management of various parameters such as pH, temperature, agitation, and others. However, the SSF approach produced better results in a comparison of the production of enzymes using the SmF and SSF methods (Premalatha et al., 2023).

1.2 Problem Statement

The demand for amylase has been increasing worldwide due to its immersion to various industries such as foods, pharmaceuticals, detergent, and textile (Farooq et al., 2021). Amylase has been stated as the second variety of enzyme employed in 90% of all liquid detergent industry. It is used to degrade starchy foods residue to dextrins and smaller oligosaccharides. Whereas, in pharmaceutical industry, amylase became prime ingredient for the medical outputs because it has various health benefits. The demand for amylase in industrial applications necessitates the optimization of production processes to ensure efficient and cost-effective synthesis of the enzyme in higher quantities. (Farooq et al., 2021).

In amylase enzyme production, the physical factors play crucial roles such as pH, temperature, agitation, incubation time and inoculum size. They are important to promote, stimulate and optimize the production of amylase. Mainly, it focusses on determining if the physical factors have the capability or causing any effect on amylase production. The physical factors, pH, temperature, agitation, incubation time and inoculum size with different value are going to be evaluated through this study.

To date this problem, identifying and selecting potential strains have a high capability for amylase production. Amylase obtained from natural sources such as soil can produce the enzyme in low amounts. Consequently, there is a need to optimize and modify microorganisms to maximize amylase production and achieve higher yields. This study is mainly focusing on the optimization of physical factors to increase the production of amylase from Isolate M. The results of this study will help develop more efficient fermentation techniques to produce more amylase, allowing businesses to fulfil the rising demand for these important enzymes.

1.3 Objectives

The two main objectives of this research are to:

1.3.1 To determine the best incubation time for the amylase production from Isolate M

1.3.2 To determine physical factors; pH, temperature, agitation, and inoculum size using Box-Behnken Design.

1.4 Scope of Study

In this study, the physical factors on amylase production from isolate M were optimized. The amylase is about to investigate the physical factors provide sufficient effect on the production of amylase. The source of amylase has been taken from the laboratory. The isolation and screening of amylase producing bacteria was done by a previous student. Before starting optimization, the amylase producing bacteria is rescreened. After that, the optimization process on amylase production will be placed and the results recorded. Moreover, the best value for each physical obtained by using Box-Behnken from Response Surface Methodology experimental design. Lastly, amylase assay was used to determine and measure the enzymatic activity. The primary source, the bacteria that produce amylase, was taken from stock culture in the previous study (Lim, 2023). The chosen amylase-producing bacteria were utilized to create an inoculum prior to refining the growing medium. For ensuing fermentation studies, the inoculum will guarantee the existence of a sufficient number of live cells.

Response Surface Methodology as an experimental design used to optimize the growth of the medium. RSM allows for the systematic variation of factors and generates response data, which can be analyzed to determine the optimal levels of the physical factors for maximum amylase production. Once optimization has completed, the study was identified and recorded the optimum physical factors. This information served as a reference for subsequent amylase production experiments.

1.5 Significance Study

Research about the effect of physical factors on amylase production has been widely investigated over the years. This study provided amylase producer from different strains through isolation and screening. The physical parameters such as pH, temperature, agitation, incubation time and inoculum size optimized to obtain the best value for the amylase production. The dintrosalicylic acid (DNS) is a simple and adoptable method to measure the amylase activity to detect or quantify the activity. My study's optimization of physical parameters such as pH, temperature, agitation, incubation time, and inoculum size demonstrate a systematic approach to maximizing amylase production, thereby increasing efficiency and yield. The use of Response Surface Methodology (RSM) as a tool for optimization adds a quantitative and rigorous aspect to your investigation, allowing for the identification of the best combination of physical factors to enhance enzyme production.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Enzyme

Enzymes are known as biologic catalysts. Enzymes could speed up the reaction rate and it will not destroy during the reaction and is used repeatedly. Most enzymes have tertiary and quaternary protein structures in the form of three-dimensional globular proteins. Every enzyme has an active site, which is a region that binds the substrate, co-factors, and prosthetic groups and contains residue that aids in holding the substrate in place. The active site occupies less than 5% of the area of enzyme. Enzymes that are present inside the cell membrane are called intracellular enzymes and used to break down the complex molecules into simpler ones, whereas the extracellular enzymes are those which are present outside the cell and performed to interact with other molecules outside of the cell (Gogate & Pandit, 2008).

Over the past 100 years, indirect uses of enzymes generated from microorganisms have been made to replace chemicals and increase the effectiveness and cost-effectiveness of a variety of industrial processes. It can be said that textile, detergent, food, medical, chemical and pulp and paper are the listed industries using enzymes in their production.

2.2 Classification of Amylase

Enzyme amylase is classified into three types, alpha, beta and gamma. They each catalyze a different location on the starch molecule, and three of them can be found in various organisms.

2.2.1 α-Amylase

In addition to microorganisms, α-amylases are present in plants, animals, and humans. Alpha amylase are calcium metalloenzymes, along with the starch chain breaks the long chain

carbohydrates. They hydrolyse the α-1,6 glycosidic linkages sectors (de Souza & de Oliveira Magalhães, 2010). It will yield maltose and maltotriose from amylose chains or glucose, maltose, and dextrin from amylopectin chains. α-amylases acts more faster than β-amylase because it can break random bonds into starch (Duan et al., 2021). Many microbes produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microorganisms that are present within the digestive tract.

Table 2.1 Amylase production produced by various microorganisms with their characterization of pH and temperature.

Microorganisms	**pH optimal/stability**	**Temperature optimal/stability (℃)**
Bacillus Subtilis	7.0	37
Bacillus Amyloliquefaciens	7.0	33
Bacillus Sp.	4.5	70
Aspergillus Niger	5.5	70
Aspergillus Fumigatus	6.0	30
Penicillium Fellutanum	6.5	30
Mucor Sp.	5.0	60
Malus Pumila	6.8	37
Penicillium olsonii	5.6	30

2.2.2 β-amylase

β-amylase is synthesized by bacteria, fungi, and plants. They produce two maltose molecules at a time by hydrolyzing the second 1,4-glycosidic link in the starch molecule. The enzyme β-amylase transforms starch into maltose during fruit ripening, giving the fruit its sweet flavor (Duan et al., 2021). Seeds contain both α-amylase and β-amylase. Mostly, β-amylase is present in seeds in inactive form prior to germination beganlase appears after germination has begun. Many microbes produce amylase to degrade extracellular starches. Animal tissues do not

contain β-amylase, although it may be present in microorganisms that are present within the digestive tract (Kumar & Chakravarty, 2018).

2.2.3 γ-amylase

γ- amylase are found in plants and animals. γ-amylase is an enzyme that is less frequently used in industry. They break the final 1,4 and 1,6 glycosidic bonds in the starch molecule to produce glucose molecules (Joshi et al., 2021). Their optimum pH is 3. They belong to the family of glycosidic hydrolases. The enzyme has a lower pH optimum compared to other amylases. They are most effective in acidic environments. The optimum pH of gamma-amylase is 3.0 (Kumar & Rastogi, 2020).

2.2.4. Endo-acting or endo-hydrolases and exo-amylase or exo-hyrolases

Exo-amylase and exo-hydrolases, as well as endo-acting and endo-hydrolases, are enzymes that break down carbohydrates, particularly starches and polysaccharides. These enzymes are essential for all species, including humans, to digest carbohydrates.

Endo-hydrolases or endo-acting enzymes have the capacity to break down the internal glycosidic linkages forming the chain of amylose or amylopectin that make up the starch (Gopinath et al., 2017). Considering their function, this kind of amylase enzyme has been given the name endoenzyme. Additionally, the endoenzyme randomly disrupts the glycosidic bond between the chains of amylose and amylopectin (Gopinath et al., 2017). Alpha- amylase enzyme is the well-known endo-amylase (Gopinath et al., 2017).

The -1, 4 glycosidic bonds and -6 glycosidic bonds found in starch can be broken down by exo-amylase or exo-hyrolases (Gopinath et al., 2017). Beta- amylase is a well-known exo-amylase that is widely used to break down the α-1, 4- glycosidic bonds of starch(Gopinath et al., 2017). Additionally, beta-amylase can act on the non-reducing end of the molecule and is also known as 1, 4-D-glucan maltohydrolase (Farias et al., 2021).

2.3 Fermentation for amylase production

Fermentation is a metabolic process in which microorganisms, such as bacteria, yeast, or fungi, convert carbohydrates (such as sugars and starches) into simpler compounds. It is an ancient technique used by humans for various purposes, including food and beverage production,

as well as in industrial processes. During fermentation, microorganisms break down the complex carbohydrates into simpler molecules, mainly through the process of glycolysis. This metabolic pathway converts glucose, or other sugars, into smaller compounds like pyruvate. In the absence of oxygen, pyruvate undergoes further reactions depending on the specific microorganism involved and the conditions of fermentation. It can be categorized into 2 main fermentation processes, solid-state fermentation, and submerged fermentation (Subramaniyam & Vimala, 2012).

2.3.1 Solid State Fermentation (SSF) for enzyme production

Solid- state fermentation can be defined as a process using microorganisms' growth on solid, non- soluble materials in absence and near absence of free water (Yafetto, 2022). The fermentation medium is generally used for growing microorganisms to receive nutritional value. This type of fermentation is an alternative for enzyme production.

The main reasons of applying sold-state fermentation are low risk of contamination, aids in increasing yield, lower energy requirement and decrease wastewater production. This solid-state fermentation is an alternative to the production in liquid submerged fermentation which has high volumetric productivity, relatively high concentration of product and require less fermentation equipment.

Solid-state fermentation is being applied in various industries for production of industrial enzymes. Production of food, pharmaceuticals, fuels, bio pesticides and some more are the listed industries. Thousands of years ago, solid-state fermentation was discovered by humans for the preparation of fermented food products (Singh et al., 2008). Then, it was improved for industrial purpose such producing industrial enzymes for production of various useful products. Almost all the known microbes can be produced under solid-state fermentation method. Enzymes of industrial importance such as protease, pectinase, cellulase, xylanase, amylases, microbial renet, tannase and oligosaccharides oxidase (Althuri et al., 2017).

2.3.2 Submerged Fermentation (SMF) for enzyme production

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. This process is mostly used to produce industrial enzymes such as protease, amylase, lipase and more. Submerged fermentation involves selected growing microorganisms, for instance bacteria and fungi, ferment in a closed vessel with high concentration of air supply, oxygen (Renge et al., 2012) .

When microorganisms are put to a closed vessel, the enzymes that are needed to break down the nutrient are released into the solution. Submerged fermentation is used mostly for various purposes, especially for industrial production. The bacterium is immersed in an aqueous solution that contains all the nutrients required for growth during submerged fermentation (Renge et al., 2012).

Industrial enzymes are excreted by microorganisms in fermentation medium to obtain the carbon and nitrogen source by breaking down. Two types of fermentation processes are common to this submerged fermentation, which are batch-fed and continuous process. In batch-fed process, the nutrients are sterilized then added to the fermenter during biomass growth. Meanwhile, the sterilized liquid nutrients are fed into the fermenter in the same amount as the broth leaving from the vessel in continuous process (Renge et al., 2012). Compared to solid-state fermentation, Smf required more space and lead to contamination easily. Submerged fermentation involves two phases, liquid and gaseous. The ease with which items can be purified is another benefit of this method (Renge et al., 2012).

2.4 Response Surface Methodology (RSM) for optimization of enzyme production

Response surface methodology, also known as response surface modelling, is a method used to optimize the response(s) when two or more quantitative factors are involved and a collection of mathematical and statistical techniques (Kumari & Gupta, 2019). Wilson in 1951 to the world. This technique involves dependent and independent variables. Dependent variables are called as response, whereas independent variable or factor as predictor variables in response surface methodology. The main objective of RSM is to obtain the optimization of response (input variable) by its chosen independent variables (output variable) (Yolmeh & Jafari, 2017).

Moreover, Response surface methodology (RSM) can be used for the approximation of both experimental and numerical responses. Mainly there are three stages in RSM, which are initial screening type is to identify few important factors from many factors, sequential search stage is for identifying the optimum design region and last stage is about the study of RSM at final stage to obtain approximation of response surface. Most frequently, Response Surface Methodology (RSM) applied in the industry area. It is crucial to industry in designing a new specific scientific study on a product. For instance, clinical, biological, social and food sciences, physical and engineering sciences are the industries utilizing RSM in their production (Kumari & Gupta, 2019).

Response Surface Methodology (RSM) is a statistical and mathematical technique used for optimizing processes and analyzing the relationship between multiple variables and their effect on a response or outcome. In the context of amylase production, RSM can be applied to optimize various factors affecting the production process, such as inoculum size, agitation, pH, temperature, and incubation time (Dinarvand et al., 2017).

2.5 Factors Affecting Amylase Production.

Both physical and nutritional factors play important roles in affecting amylase production. Here are some specific factors within each category.

2.5.1 Physical Factors

2.5.1.1 pH

As a main physical factor, pH is one of the important in the production of amylase and it influences enzyme activity. Each type of amylase has its own pH level to optimize the enzyme activity. Although, there are alkaline and acidic amylase used in the industries depends on the utilization. The purified enzyme is optimally active at pH 5.0. Usually, the acidic amylase is produced actively with the pH range between 3.0 to 6.0 (Apostolidi et al., 2020).

Alkaline amylase is stable and effectively hydrolyze the starch under alkaline conditions (Yang et al., 2012). The best optimum pH for the alkaline amylase was the range of 7.0 to 11.0. Alkaline amylase is widely used in detergent, textile, pharmaceutical and other industries.

Neutral amylase, which means at pH level 7.0 is the pH for amylase. This is because the starch broke down more quickly at the pH of 7.0. In this study, different pH values are applied to the amylase production from Isolate M. The values are from 6 to 8 pH level (Dash et al., 2015).

2.5.1.2 Temperature

Another critical factor that affects amylase production is the temperature. The stability of the enzyme always depends upon the temperature. The activity of amylase was influenced by temperature and determined it by incubating the fermentation media on different temperature at different temperatures and the further assayed was under standard conditions for the activity of enzyme produced by *B. licheniformis* strain 2618 utilizing 1% starch. It has been mentioned by author that the majority of bacterial amylase have its optimum temperature range between 30°C to 100°C (Divakaran et al., 2011). A study found that the amylase activity was effective at the temperature 37°C when compared to 35°C (Asgher et al., 2007). Activity of α-amylase significantly decreased with the increase in the temperature beyond 37°C. The growth of microbes can be inhibited at one temperature, but it can also be activated at another temperature (Divakaran et al., 2011). This is because each microorganism has its specific and optimum temperature for cell growth and amylase production (Divakaran et al., 2011) .

2.5.1.3 Inoculum size

Optimization of inoculum size should always be considered as an important fermentation parameter. The size of inoculum plays a significant role in the fermentation process especially for amylase production (Simair & Mangrio, 2017). A study found that the amylase from Bacillus sp. has a better production at the inoculum size at 10 % (v/v) at 24 hours of fermentation (Simair & Mangrio, 2017). Different sizes of inoculums (0.5, 1, 2, 2.5 and 3% v/v) were tried to determine the effect on alpha from Bacillus sp. for amylase production. The study achieves that the optimum inoculum size for the production was 2.5% v/v which was fermented for 2 days. Meanwhile, in 0.5% inoculum size occurred minimum production of α-amylase which was observed for 3 days. As the level of inoculum was further increased, the productivity of α-amylase was decreased (DemİRkan et al., 2017).

2.5.1.4 Agitation

Agitation is another factor that affects the production of enzymes. Agitation refers to the mechanical stirring or shaking of the fermentation media during the incubation process (Zhou et al., 2018). Agitation is commonly used in fermentation processes to ensure uniform mixing of the culture broth, distribute oxygen, and prevent the settling or clumping of cells or particles. Through constant mixing, it not only enhances mass and oxygen movement between the various phases but also keeps the medium's chemical and physical properties uniform (Zhou et al., 2018). A study found that the maximum of α-amylase production and has a better bacteria growth were determined at 150 rpm. It shows a decrease in production when the agitation speed is set at 200 rpm. Additionally, it obtained the lowest amount of α-amylase production at 50 rpm. Thus, it was considered 150 rpm is the best optimum agitation for amylase production for this production (DemİRkan et al., 2017).

2.5.1.5 Incubation time

The incubation time refers to the duration for which a sample or culture is kept under specific conditions, such as temperature, humidity, and other environmental factors, to allow for desired processes to occur. Most of the study investigated the study with different incubation time such as 12h, 24h, 36h, 48h, 60h and 72h to determine the best for the production (Henshaw & Wakil, 2019). The study showed an optimal growth and amylase production at the range of 36 hours to 48 hours of incubation time for the *Bacillus spp*. Further increase in incubation time, resulted in reduced growth and amylase activities (Henshaw & Wakil, 2019). Initially, an extended incubation time may allow more time for microbial growth and enzyme production, leading to higher levels of amylase activity. However, beyond a certain point, prolonged incubation can lead to nutrient depletion, accumulation of metabolic byproducts, and changes in environmental conditions such as pH and temperature. These adverse conditions can negatively impact microbial viability and metabolic activity, resulting in decreased growth and enzyme production (Naghshbandi et al., 2019).

2.5.2 Nutritional Factors

2.5.2.1 Carbon and nitrogen source

Microorganisms require a source of carbon for energy and growth, and nitrogen for protein synthesis. Suitable carbon sources, such as glucose, maltose, or starch, are utilized by microorganisms to produce energy and provide the building blocks for amylase synthesis. Meanwhile nitrogen sources, like peptones, yeast extract, or ammonium salts, are essential to produce enzymes and other proteins. The production of amylase has been assessed and optimized using a variety of carbon sources, including lactose, sucrose, maltose, and the monosaccharides xylose, galactose, and fructose. According to reports, most of the *Bacillus sp.'* production of a carbohydrate-degrading enzyme results in catabolic suppression when readily metabolizable substrates like glucose and fructose are present (Sudharhsan et al., 2007).

2.5.2.2 Micronutrients

Micronutrients are essential for the growth and metabolism of microorganisms. They act as cofactors or activators for enzymes, including amylase. For example, calcium ions are often required for the stability and activity of amylase. Other micronutrients like magnesium, zinc, and various B vitamins are also important for optimal enzyme production. These metal ions often bind to specific sites on enzymes and play crucial roles in stabilizing enzyme structure, facilitating substrate binding, and catalyzing chemical reactions. The addition of Mg2+ and Ca2+ boosted the synthesis of α-amylase among the metal ions that were added to the fermentation medium (Sethi et al., 2016). Additionally, phosphorus and sulfur are essential micronutrients for enzyme production due to their critical roles in various cellular processes. Phosphorus, metal ions, and sulfur are indispensable micronutrients for enzyme production because they participate in energy metabolism, enzyme structure stabilization, substrate binding, catalysis, and regulatory processes within cells (Sethi et al., 2016).

2.6 Industrial Application of Amylase

Amylase has found numerous applications in various industrial sectors such as and industries, fuel alcohol industries, textile industries, food industry and in paper industries. These

enzymes have been employed in a wide array of applications for many years with satisfactory results. Because of the wide application in industries, this enzyme is anticipated to reach at value CAGR of 5.9% and estimated to reach US$ 2,692.5 million by the end of 2033. In the year of 2012, the demand and value for amylase was US$ 2.7 billion (Elmarzugi et al., 2014).

2.6.1 Detergent industry

The largest and most valuable consumers of enzymes are the detergent industries. The main purpose of using enzymes in detergent production is to improve the formulation of detergent in removing tough stains and make the product safe to environment (Niyonzima & More, 2014). 90% of all liquid detergents include amylase in their production and amylase known as second type of enzyme utilizing in the detergent formulation (Gupta et al., 2003). Amylases are one of the most important industrial enzymes having approximately 25% of enzyme market particularly in detergent industry.

The amylases are added to the detergent precisely to help it break down starchy stains. Amylase is active at lower temperature and its pH is alkaline (de Souza & de Oliveira Magalhães, 2010). It supports the essential stability of amylases in detergent conditions and their oxidative stability. Removal of starch provides the whiteness benefit to the clothes. Examples of amylases from Bacillus species or Aspergillus species that are utilized in the detergent business (Patil et al., 2021).

2.6.2 Fuel Alcohol Production

As an important and most utilized biofuel is made of ethanol. For ethanol production, starch is used as a substrate because of its low price in the market and easily available as raw material in most regions of the world (Kirk et al., 2002). During this production of fuel, the starch obtained must be solubilized and then transferred to two enzymatic steps to produce fermentable sugars. Once the starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase, then fermentation process for the fuel production will occur. In this fermentation, the sugar will be converted into ethanol using ethanol fermenting microorganisms such as yeast *Saccharomyces cerevisiae* (de Moraes et al., 1999). Among bacteria, α-amylase obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is utilized during the first step of starch suspensions hydrolysis (Sánchez & Cardona, 2008)

2.6.3 Textile Industries

Amylase plays an essential part in textile industry for wet process as replacement for chemicals. Amylase is mainly used for desizing process (Kalia et al., 2021). Starch as sizing agents applied to yarn in textile production for fasting and secure weaving process. Due to the availability of starch in most regions of the world, the demand for starch is highly mentionable. In the textile finishing industry, starch is afterwards taken out of the woven cloth using a wet process. Desizing is the process of removing the starch from the fabric, which acts as a strengthening agent to stop the warp thread from breaking during the weaving process. The α-amylases eliminate size in a selective manner and do not harm the fibres. Amylase from Bacillus sp. strain was employed in textile industries for quite a long time (Saini et al., 2017)

2.6.4 Food Industries

Many different food industries, including those that produced cakes, brew beer, provide digestive aids, made fruit juices, and made starch syrups, have used amylase extensively (Couto & Sanromán, 2006). Compared to other food industries, the baking industry is widely using amylase in their production (Raveendran et al., 2018). Adding amylase to flour can enhance fermentation rate, reduce viscosity, and improve volume and texture of bread by breaking down starch into smaller dextrins for yeast fermentation. In addition, amylase generates natural and additional sugar to the bread which helps to improve the taste, crust colour and toasting quality of bread.

In addition to producing fermentable chemicals, amylase has an anti-staling effect while baking bread and enhances the softness retention of baked goods, extending the shelf life of these goods (Gupta et al., 2003). In addition to being employed in the baking sector, amylases are also used in the clarifying of fruit juices, beer, and animal feed to increase fibre digestibility (Vitolo, 2020).

CHAPTER 3

MATERIALS AND METHOD

3.1 Apparatus

Micropipette, cuvette, vortex, beakers, conical flask, spectrophotometer, incubator, laminar flow chamber, pipette tip, water bath, autoclave, incubator shaker, pH meter, balance, inoculating loop, test tubes, screw cap test tubes, petri dish, centrifuge tube, dropper, Bunsen burner and Schott bottles.

3.2 Materials

Distilled water, starch agar powder, gram's iodine, DNS solution (3, 5 -dinitrosalicyclic acid), ethanol, starch solution, magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), yeast extract, fresh nutrient agar powder, soluble starch, ammonium sulphate $(NH_4)_2SO_4$, monopotassium phosphate (KH_2PO_4), calcium chloride dihydrate ($CaCl_2.2H_2O$), sodium phosphate buffer, Tris-HCI and maltose powder.

3.3 Bacterial Strain

Isolate M was isolated from soil sample (Lim S. E ,2023). The isolate M was taken from glycerol stock at -80℃ in Microbial Technology Lab.

3.4 Method

3.4.1 Screening Amylase Producing Bacteria Using Starch Hydrolysis Test

A single colony of isolate M using subcultured on starch agar medium by using sterile inoculating needles. These plates then incubated at 37°C for 48 hours. After incubation, the plates floated with Gram's Iodine to test the starch hydrolysis. Formation of clear zone around the colonies observed and the diameters measured using a ruler. The clear zone ratio calculated using the following formula,

$$\text{Clear zone ratio} = \frac{\text{Diameter of clear zone (mm)}}{\text{Diameter of colony (mm)}}$$

Equation 3.1

3.4.1.1 Preparation of Starch Agar

1 % (w/v) starch agar medium weighed and added into 1 liter of distilled in conical flask. Then, the mixed solution and autoclaved it at 121℃ for 15 minutes. Then, poured it on petri dishes.

3.4.2 Inoculum preparation

The bacterial isolates streaked on separate fresh nutrient agar medium. The plates incubated at 37℃ for 24 hours. A loop full of the freshly grown colony inoculated in separate screw cap test tubes containing a nutrient agar medium. Then the test tubes incubated at 37℃ for 3 to 5 hours.

3.4.3 Optimization of physical factors on amylase production

The experiment was conducted by using submerged fermentation, submerged fermentation uses liquid media as substrate. The media prepared using the following composition: 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 $CaCl_2.2H_2O$. Inoculum 2 %, 5 % and 8 % (v/v) inoculated into 250ml of Erlenmeyer flask containing 100ml of fermentation media. The pH (sodium phosphate buffer &Tris- HCl) of the media will be adjusted differently, and the media will be incubated at different incubation times, different temperatures, and different agitation speed on shaking incubator as listed below in the Table 3.1. After incubation, the crude enzyme is extracted for amylase assay.

Table 3.1 Experimental variable at different levels used to produce amylase

VARIABLES	UNITS	Actual variables at coded variable levels		
		Low (-1)	Centre (0)	High (1)
Agitation	rpm	100	150	200
Temperature	°c	30	35	40
pH		6	7	8
Inoculum size	%	2	5	8

Table 3.2 Optimization of fermentation parameters using the RSM with the Box-Behnken Design (BBD)

Std	Run	Agitation (rpm)	Temperature (°C)	pH	Inoculum size (%)
22	1	150	40	7	2
16	2	150	40	8	5

5	3	150	35	6	2
25	4	150	35	7	5
9	5	100	35	7	2
15	6	150	30	8	5
19	7	100	35	8	5
1	8	100	30	7	5
14	9	150	40	6	5
24	10	150	40	7	8
3	11	100	40	7	5
8	12	150	35	8	8
13	13	150	30	6	5
29	14	150	35	7	5
26	15	150	35	7	5
28	16	150	35	7	5
7	17	150	35	6	8
6	18	150	35	8	2
11	19	100	35	7	8
10	20	200	35	7	2
18	21	200	35	6	5
12	22	200	35	7	8
21	23	150	30	7	2
4	24	200	40	7	5
17	25	100	35	6	5

2	26	200	30	7	5
20	27	200	35	8	5
23	28	150	30	7	8
27	29	150	35	7	5

3.4.4 Extraction of crude enzyme

After incubation, 10 ml of broth was collected. The broth then centrifuged at 6000 rpm for 10 minutes. The supernatant obtained was the crude enzyme. It was stored at 4 °C until use.

3.4.5 Response Surface methodology (RSM)

RSM was used in this study to optimize the variables according to their culture conditions and parameters. Optimization of this study using RSM that complements the combination of Box-Behnken Design (BBD). Response surface designs called Box-Behnken designs can accommodate a full quadratic model. 29 runs of experimental design were executed to get the 3D Surface result. The independent variables are agitation, incubation time, temperature, pH, and inoculum size. After identifying the optimal incubation time, they optimized the optimum levels of pH, temperature, agitation, and inoculum size. Each variable will be studied at different levels (-1, +1) and center point (0). The minimum, center and maximum ranges of variables investigated which the minimum pH is 6, center 7 and maximum is 8. Whereas the temperature will set minimum 30°C, center is 35°C and maximum will be 40°C. While minimum agitation is 100 rpm, center is 150 rpm, and 200 rpm were set as the maximum. The inoculum size, minimum is 2%, center 5% and maximum is 8%.

3.4.6 Amylase Assay

1.0 mL of starch in buffer added into the 1.0mL crude enzyme. After that, incubate it at the best temperature obtained from the temperature test, in water bath for 30 minutes. Then, added 2.0 mL of DNS reagent (3, 5 -dinitrosalicylic acid) into solution and continue incubating for 5 minutes in 35℃ water baths. After incubation, cooled down the solution at room temperature. The absorbance of blank substrate measured at 540 nm in spectrophotometer then proceeds with all the sample containing brown reduction product. The absorbance reading

recorded. The enzyme activity was measured according to the DNS method based on the standard maltose curve.

3.4.7 Formation of Glucose Standard Curve

To separate test tubes, different concentration of glucose solutions was added. The concentrations decided according to the following composition illustrated in Table 3.3.

Table 3.3 Amount of components used to formulate the glucose standard curve

Concentration of Glucose (ug/ml)	Mass of glucose (mg)	Volume of DNS (ml)	Reading of Abs
0	0	1	-
100	0.1	1	-
200	0.2	1	-
400	0.4	1	-
800	0.8	1	-
1000	1.0	1	-

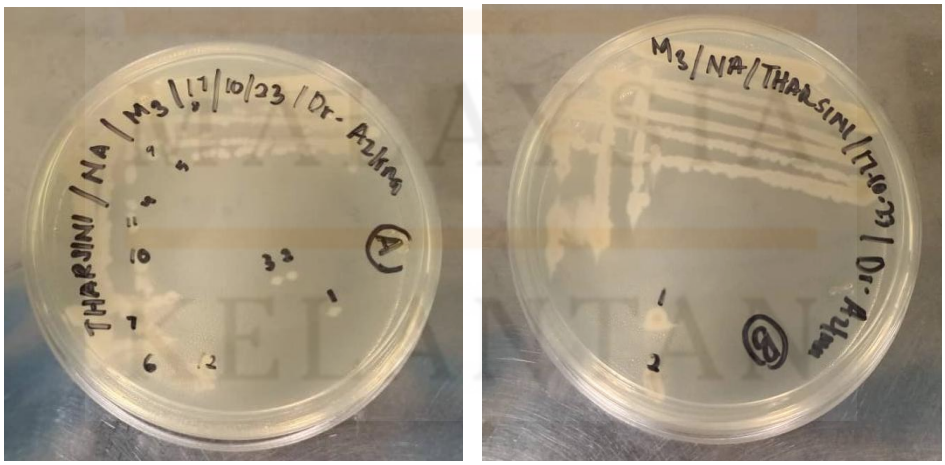
To each concentration, 1 ml of DNS solution is added. The test tubes were boiled at 100°C for 10 minutes in a water bath. Then, cooled them at room temperature. The intensity of color of each solution measured in absorbance value in a spectrophotometer at 540 nm. Then, plotted the graph with the amount of glucose along the x-axis and the reading of abs along y-axis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Screening of Amylase Produce Bacteria from Isolate M

In this study, the isolate M was obtained from the local stock culture in Microbial Technology Lab, UMK (Lim, S. E. (2023). Then, streaked the stock culture onto seven nutrient agar plates, creating single colonies on each plate. The nutrient agar plates were then incubated at a temperature of 37 ℃ for a duration of 24 hours. The incubation period allowed for the optimal growth conditions for the bacteria, promoting the development of distinct colonies on the agar surface. Following the incubation period, the plates were observed, revealing that only three out of the seven plates exhibited bacterial growth as shown in Figure 4.1. Subsequently, the experiment proceeded to the next phase of the experiment by selecting single colonies from the plates that exhibited bacterial growth. The selection of single colonies is crucial for ensuring the isolation of pure bacterial strains.



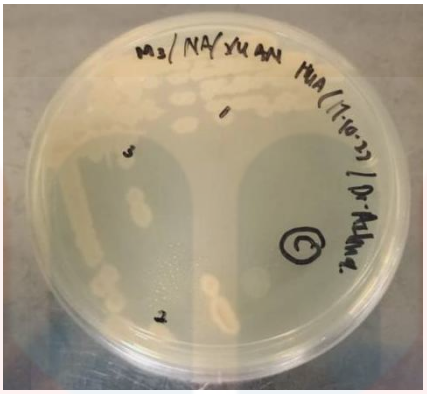


Figure 4.1: Grown single colonies labelled with numbers on nutrient agar plate A, B and C.

4.2 Screening and selecting potent Amylase Produce Bacteria using Starch Hydrolysis Test

The single colonies which were selected then subcultured on 1% (w/v) starch agar medium using single streaking technique. Each plate has been screened with two different colonies, selected from the A, B and C plates respectively, aimed to assess the ability of these colonies to utilize starch as a carbon source. After that, those three plates were incubated at 37 ℃ for 24 hours in the incubator. After the subculturing process, the newly streaked starch agar plates were incubated at a temperature of 37 ℃ for a period of 24 hours. The incubation allowed for the evaluation of bacterial growth and the formation of observable colonies on the starch agar medium. Notably, the plates revealed the presence of single-line colony growth after the 24 hour incubation period. These three plates then flooded with Gram's Iodine solution to observe the starch hydrolysis. Once the solution on these plates showed a positive result on all the six plates with presence of clear zone around the grown single line colony. Gram iodine is used on starch agar plates to test starch hydrolysis due to its ability to form a complex with starch. The presence of starch iodine forms a complex with the starch molecules, resulting in a color change, which appears as dark blue. Because amylase is present, the clear zone that surrounds colonies shows that microorganisms can break down the starch in the medium (Lal & Cheeptham, 2012). Furthermore, the 6 colonies from A2, C1, A10, A6, C2 and A4 which have showed positive result were chosen for studying clear zone ratio as shown in the Figure 4.2

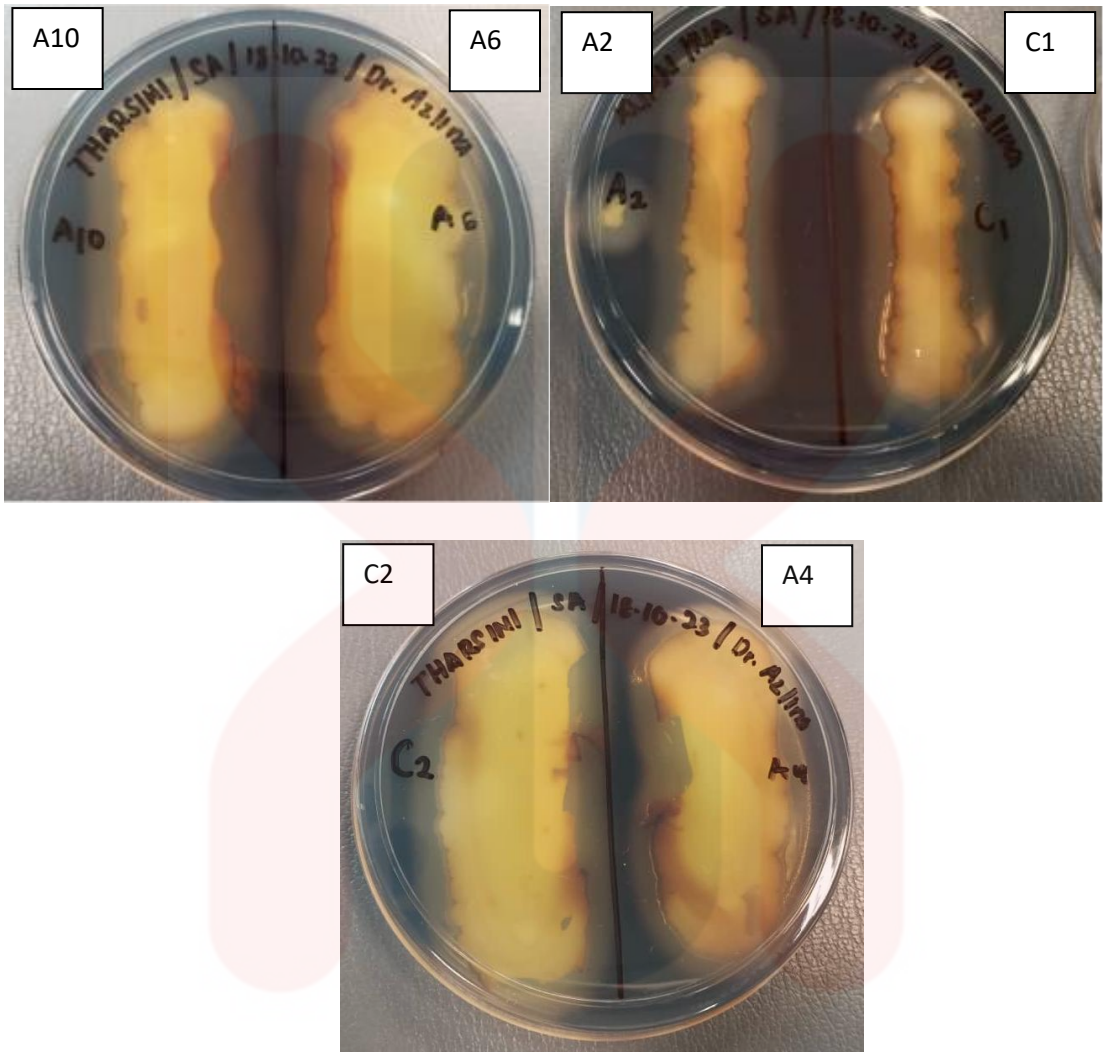


Figure 4.2 A2, C1, A10, A6, C2 and A4 of the presence of clear zone around colonies

Moreover, the six plates which have formed clear zone around the colonies were observed and measured the diameter using ruler. A larger clear zone diameter denotes a higher level of bacterial hydrolysis activity. This is because of the bacteria producing more amylase and hydrolyzing more starch in the agar medium. Table 4.1 shows the diameter of chosen six strains on starch agar plates.

Table 4.1: Ratio of clear zones of selected colonies on starch agar media shows the largest clear zone.

No	Isolate M substrain	DIAMETER OF CLEAR ZONE (mm)	DIAMETER OF COLONY (mm)	CLEAR ZONE RATIO
1	A2	6	21	0.29
2	C1	5	22	0.23
3	A10	8	29	0.26
4	A6	5	33	0.15
5	C2	6	34	0.18
6	A4	5	32	0.16

The results from Table 4.1 indicate that isolated M substrain A2 exhibited the most significant starch hydrolysis, as evidenced by the largest clear zone (0.29mm) around its colony. This suggests a robust amylolytic activity in isolate M substrain A2 compared to the other tested isolates. The clear zone size provides valuable information about the efficiency of starch degradation by each isolate.

4.3 Optimization of best incubation time for amylase production from Isolate M

The best isolate M selected to proceed with streaking on nutrient agar plates. The plates were incubated at 37 ℃ for 24 hours for observing single colony growth. After 24 hours incubation, the single colony was successfully produced without any overgrown on the plates. Then, the single colony plates were proceeded with inoculation process. This inoculation has been prepared to optimize the best incubation time for amylase producer which has to be decided to apply when conducting Response Surface Methodology. The inoculum size, agitation, pH, and temperature are fixed variable for this fermentation media which are 2%, 150 rpm, 7 and 35℃ respectively. But the fermentation media was observed with different incubation times, 24 hours, 48 hours, and 72 hours.

Before proceeding with fermentation, inoculum was prepared using nutrient broth as seed media. All the necessary nutrients, including vitamins, carbohydrates, amino acids, and many more, are present in the nutrient broth. The growth of microorganisms at the proper temperature depends on these nutrients. The inoculum preparation was done by inoculating one loop of single colony from isolate M plate into the nutrient broth. The inoculum was incubated for 18 hours at 37°C in shaking incubator. After incubation, the inoculum was undergoing the optical density test through spectrophotometer at OD_{600} = 0.5nm. Turbidity of the nutrient broth was observed after incubation. The turbidity of nutrient broth with inoculum refers to the cloudiness or haziness of the liquid medium resulting from the introduction of microorganisms. Measuring turbidity is the method to estimate the growth of microorganisms in a nutrient broth and quantified using a spectrophotometer, increased turbidity corresponds to higher microbial cell density in the nutrient broth. After successfully obtained OD at 0.5 nm, the inoculums were further fermented for optimizing the best incubation time for the amylase production. The broth cultured were taken for each 24 hours interval.

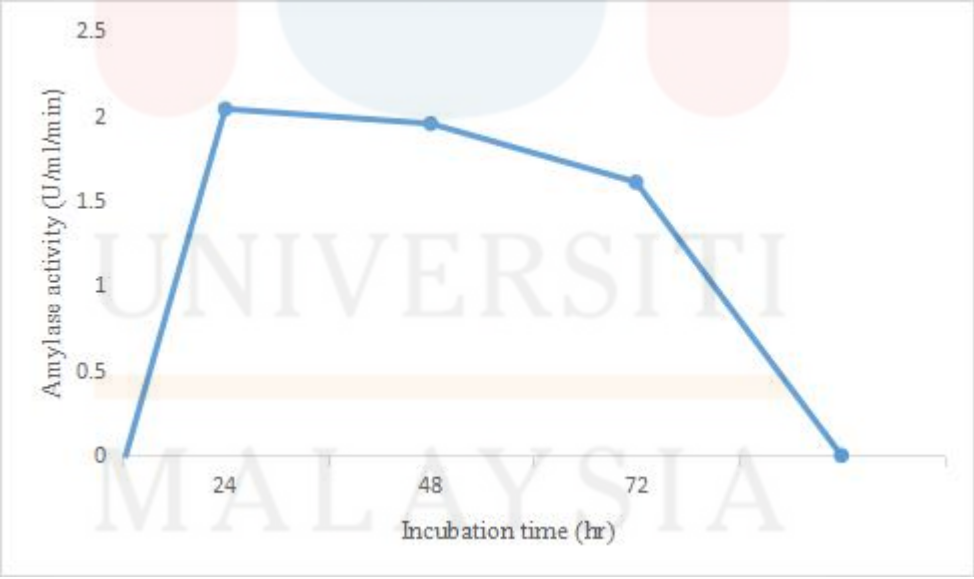


Figure 4.3 Incubation time for amylase production within 72 hours

In submerged fermentation (SmF) method, incubation time played a crucial role as a major factor in the production of amylase. After fermenting for 24 hours to 72 hours, the results were obtained that 24 hours was the best optimum incubation time for production. Three of the incubation time obtained the amylase activity, which 2.04, 1.95 and 1.61 (U/mL) respectively. By this result, it can be seen clearly that 24 hours has been fermented well and has the better capability of produce highest number of amylase activity. Then, 24 hours of incubation time has fixed as fixed variable. Therefore, the incubation time was used 24 hours for the whole optimization process. A study stated that 24 hours of incubation was achieved the maximum production (Salman et al., 2016). In this study, the lag phase occurred initially, as bacteria adapted to the growth medium. The period from 24 to 72 hours represents the log phase, characterized by exponential growth and increasing amylase activity. Following this, a stabilization or slight decrease in amylase activity indicates the onset of the stationary phase, where growth rate equals cell death rate.

4.4 Optimization of physical factors on amylase production from Isolate M

For the effect of physical factors on amylase production, four independent variables were observed which are pH, inoculum size, temperature, and agitation through response surface methodology (RSM). The level of independent variables used only three with coded 1, 0, and -1. The pH value was measured with 6,7 and 8. Agitation was at 100, 150 and 200 rpm whereas the temperature was measured with 30℃, 35℃ and 40℃. While inoculum size was 2%, 5% and 8%. The highest activity of amylase was observed 57.65 U/mL.

Maximum amylase production was obtained under the temperature of 35 ℃, pH 7, agitation at 150 rpm and inoculum size was 5%. The highest activity of amylase was 57.65 U/mL while the lowest reached 2.22 U/mL. The lowest yield was under temperature of 30℃, pH 7, agitation 150 rpm and inoculum size 2%. Even though the pH and agitation were at same level, the effect was received from inoculum size and temperature. The most significant factors for amylase production are incubation time, temperature, inoculum size, agitation, and chemical factors (Dar et al., 2015). For this experiment, pH, temperature, agitation, and inoculum size played important roles in the amylase production.

Table 4.2 Optimization of Fermentation Parameters using the RSM with the Box-Behnken Design (BBD)

Std	Run	Agitation (rpm)	Temperature (°C)	pH	Inoculum size (%)	OD 540nm value	Response: Amylase activity (U/mL)	Predicted amylase activity (U/mL)
22	1	150	40	7	2	0.431	31.91	24.48
16	2	150	40	8	5	0.087	6.45	20.51
5	3	150	35	6	2	0.099	7.32	20.46
25	4	150	35	7	5	0.101	7.47	16.49
9	5	100	35	7	2	0.173	12.80	15.75
15	6	150	30	8	5	0.114	8.43	6.89
19	7	100	35	8	5	0.187	13.84	11.77
1	8	100	30	7	5	0.247	18.28	7.75
14	9	150	40	6	5	0.598	44.25	26.08
24	10	150	40	7	8	0.305	22.57	22.11
3	11	100	40	7	5	0.277	20.50	21.37
8	12	150	35	8	8	0.074	5.47	12.51
13	13	150	30	6	5	0.088	6.51	12.46
29	14	150	35	7	5	0.182	13.47	16.49
26	15	150	35	7	5	0.088	6.51	16.49
28	16	150	35	7	5	0.779	**57.65**	16.49

7	17	150	35	6	8	0.119	8.80	18.09
6	18	150	35	8	2	0.080	5.92	14.89
11	19	100	35	7	8	0.177	13.01	13.37
10	20	200	35	7	2	0.330	24.42	19.60
18	21	200	35	6	5	0.187	13.84	21.20
12	22	200	35	7	8	0.179	13.24	17.23
21	23	150	30	7	2	0.030	**2.22**	10.86
4	24	200	40	7	5	0.281	20.79	25.22
17	25	100	35	6	5	0.332	24.57	17.35
2	26	200	30	7	5	0.298	22.05	11.60
20	27	200	35	8	5	0.429	31.75	15.63
23	28	150	30	7	8	0.098	7.25	8.49
27	29	150	35	7	5	0.091	6.73	16.49

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

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

There are four 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 6 – 58 U/ml, which considered quite a big gap of range.

The analysis of variance, ANOVA is crucial to test significance and adequacy of the model F- test value is the ratio between the mean square of the model and the residual error (experimental error) perform this comparison. As shown in table 4.3, the Model F-value of 1.15 implies the model is not significant relative to the noise. There is a 35.91% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.18 implies the Lack of Fit is not significant relative to the pure error. There is a 99.65% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good.

Table 4.3 ANOVA of Box-Behnken Design (BBD) for the Linear Model for amylase production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	711.25	4	177.81	1.15	0.3591	not significant
A-Agitation	44.50	1	44.50	0.2867	0.5972	
B-Temperature	556.69	1	556.69	3.59	0.0703	
C-pH	93.17	1	93.17	0.6004	0.4460	
D-Inoculum size	16.89	1	16.89	0.1088	0.7443	
Residual	3724.38	24	155.18			
Lack of Fit	1762.47	20	88.12	0.1797	0.9965	not significant
Pure Error	1961.91	4	490.48			
Cor Total	4435.63	28				

4.5 Interaction among variables for amylase production from Isolate M

Contour plots in the software are used to explain the comparative effects of two different variables while holding the other factors fixed at the center of the plot.

4.5.1 Interaction effects between agitation and temperature on amylase production

The response interacted in figure 4.7 showed that the function of amylase activity between temperature and agitation. The center of the contour plot explained the interaction of these two factors, and which is the best value are respective with another two factors inoculum

size and pH. Therefore, the best values are temperature 30℃ and agitation at 100 rpm. Interaction among factors is perfect with each of these. The red color on the contour represents the highest amylase activities value.

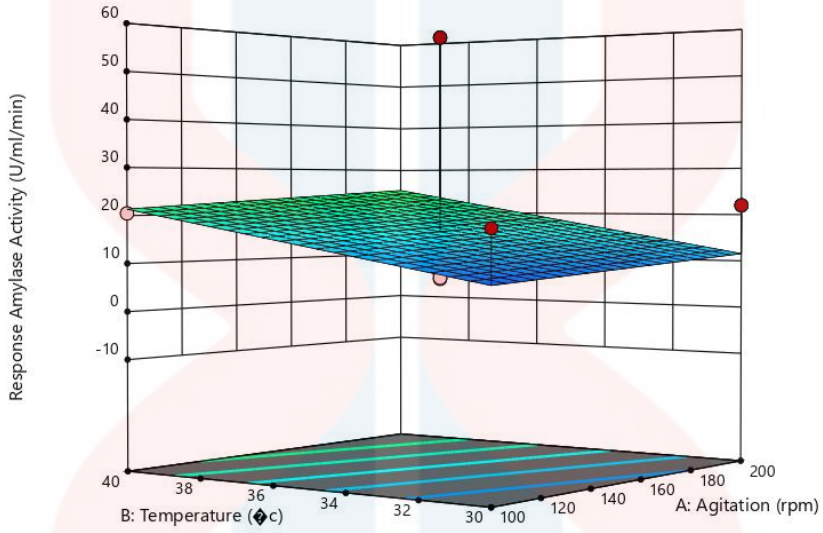


Figure 4.7 Response Surface plot showing the interactions between temperature and agitation on amylase activity.

4.5.2 Interaction among agitation and pH on amylase production

Figure 4.8 showed the effect of agitation and pH on the production of amylase. At the values of agitation at 150 rpm and pH 7, the optimum activity of amylase production was obtained. The moderate agitation level of 150 rpm likely contributes to an optimal mixing of the culture medium. The pH of 7 is known to be a favorable condition for many amylase-producing microorganisms. The case of amylase, a pH of 7 is neutral, and many microbial amylases exhibit optimal activity under neutral or slightly alkaline conditions.

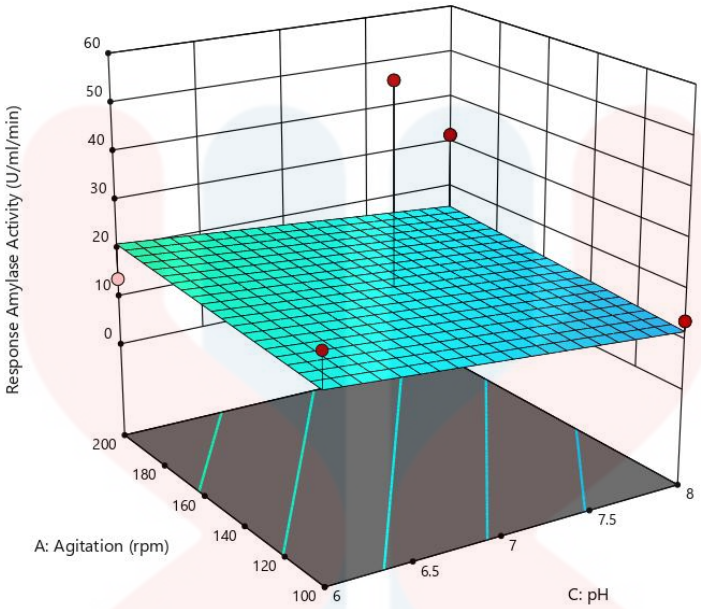
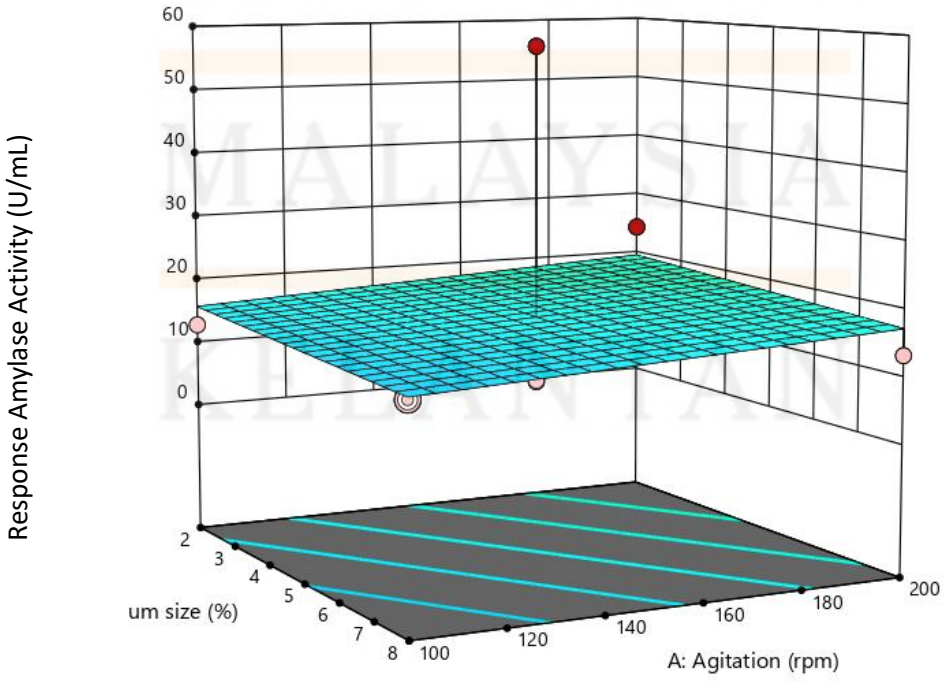


Figure 4.8 Response surface plot showing the interactions between pH and agitation on amylase activity.

4.5.3 Interactions between inoculum size and agitation on amylase production

At the moderate level of agitation 150 rpm and 5% of inoculum size, has reached the highest production of amylase. Whereas the amylase activity was low at the 8% of inoculum size used along with the agitation at 100 rpm. The reduced agitation level might have led to uneven nutrient distribution, limiting microbial growth, and subsequently impacting amylase production.



D: Inoculum size (%)

Figure 4.9 Response surface plot showing the interactions between inoculum size and agitation on amylase activity.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the incubation time and optimising the physical factors that affect the amylase production are achieved to produce higher production of amylase from Isolate M.

In this study, a Box-Behnken design Response Surface Methodology was employed to investigate the impact of various physical factors on amylase production. The results revealed significant interactions among four key factors in the production process. Utilizing response surface plots, the study aimed to estimate the interplay of agitation, temperature, pH, and inoculum size on the response variable, namely amylase activity. A comprehensive mathematical analysis was conducted based on the experimental data derived from 29 samples. Through optimization across these samples, each with distinct factor levels, it was determined that the combination of pH 7, agitation at 150 rpm, 5% inoculum size, and a temperature of 35°C yielded the maximum amylase activity, reaching 57.65 U/mL.

5.2 Recommendation

As a recommendation, a further study is needed to improve the production of amylase with more different levels of variables and more different independent variables to make the production multiple. Experimental design applications must be more versatile such as response surface methodology to explore and treat problems with large numbers. For the optimization of amylase production, a multifaceted approach is recommended, considering key factors such as incubation time, agitation, temperature, inoculum size, and pH. Employing a systematic investigation using Response Surface Methodology (RSM) can help unravel the complex interactions among these variables. A suggested strategy involves conducting a series of experiments, varying each factor within a specified range, to observe their collective impact on amylase activity. The optimal conditions can be identified by analyzing response surface plots generated from the experimental data. It is advisable to explore a range of incubation times, agitation speeds, temperatures, inoculum sizes, and pH levels to comprehensively assess their influence on amylase production. Fine-tuning these parameters through experimental design will enable the development of an optimized protocol for enhanced amylase yield, catering to the specific needs of industrial applications in Malaysia or elsewhere. This systematic and data-

driven approach ensures a thorough understanding of the interplay between these factors, leading to the production of high-quality amylase enzymes tailored for industrial demands.

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APPENDIX A

1. Nutrient agar media (1L)

28g of nutrient agar powder dissolve in 1000 mL distilled water and swirl to mix. Then, autoclave.

2. Starch agar media (1L)
   25 g starch agar powder dissolve in 1000mL distilled water and swirl to mix. Then, autoclave.
3. Fermentation media (50 mL)
   0.25 g soluble starch, 0.25 g yeast extract, 0.125 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g KH_2PO_4 and 0.0125 g are dissolved into 50ml distilled water. Then, the 250 ml Erlenmeyer flask that contained fermentation liquid media was autoclaved.

APPENDIX B

Figure B Experimental variable at different levels used to produce amylase.

VARIABLES	UNITS	Actual variables at coded variable levels		
		Low (-1)	Centre (0)	High (1)
Agitation	rpm	100	150	200
Temperature	°c	30	35	40
pH		6	7	8
Inoculum size	%	2	5	8

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Figure B Amount of components used to formulate the maltose standard curve

Concentration of Glucose (ug/ml)	Mass of maltose (mg)	Volume of DNS (ml)	Reading of Abs
0	0	1	0.000
100	0.1	1	0.256
200	0.2	1	0.452
400	0.4	1	1.021
800	0.8	1	2.134
1000	1.0	1	2.347

APPENDIX C

Figure C Glucose standard curve