OPTIMIZATION OF AMYLASE REACTION THROUGH DESIGN OF EXPERIMENT (DOE)

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Special Dedication to my beloved family members,
lecturers, my fellow colleagues,
and all faculty members.

For all your care, support and believe in me.

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ABSTRACT

The uses of starchy biomass as an industrial raw material bring commercial success of amylases, which have potential industrial applications due to the ability to hydrolyze starch to glucose. The implementations of conventional method that employ screening using one factor at a time usually give inaccurate optimum condition. Moreover, this method is time consuming. Thus, this paper aims to apply design of experiment as a tool to optimize enzymatic reaction. In this work, the two-level full factorial design and response surface methodology with central composite design was used to identify and optimize the significant parameters. The experiments were conducted with the aid of Design Expert Software 7.1.6. The effect of pH, temperature, substrate concentration and enzyme concentration were studied. The results concluded that the optimized value for pH, temperature and substrate concentration were 8.90, 40.76 °C and 5.46% (w/v), respectively; with R^2 of 81.86%. The experimental design was proven to be tool for optimization process with less number of experiments and more accurate optimum value.

ABSTRAK

Penggunaan biojisim yang menggunakan kanji sebagai bahan mentah membawa kelebihan komersil kepada amilase, kerana kemampuan amilase untuk menghidrolisis kanji kepada glukosa. Pelaksanaan kaedah konvensional yang menggunakan penyaringan satu faktor pada satu masa biasanya memberi keadaan optimum yang tidak tepat. Selain itu, kaedah ini juga memakan masa. Oleh itu, kajian ini bertujuan untuk menggunakan rekabentuk eksperimen sebagai cara untuk mengoptimumkan tindak balas enzim. Di dalam kajian ini, rekabentuk penuh faktorial dua peringkat dan kaedah permukaan sambutan (RSM) dengan rekabentuk komposit pusat (CCD) digunakan untuk mengenalpasti dan mengoptimumkan parameter yang signifikan. Eksperimen ini dilakukan dengan bantuan perisian *Design Expert* versi 7.1.6. Pengaruh pH, suhu, kepekatan substrat dan kepekatan enzim dikaji. Secara kesimpulannya, nilai optimum untuk pH, suhu dan kepekatan substrat adalah 8.90, 40.76 °C and 5.46% (w/v), masing-masing; dengan R^2 81.86%. Rekabentuk eksperimen terbukti boleh digunakan sebagai cara untuk proses pengoptimuman dengan jumlah eksperimen yang berkurangan dan nilai optimum yang lebih tepat.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
RSM	-	Response surface methodology
CCD	-	Central composite design
DNS	-	Dinitrosalicylic
ES	-	Enzyme-Substrate complex
DOE	-	Design of experiment
V_{max}	-	Maximum velocity of reaction
g	-	Gram
g/l	-	Gram per Liter
w/v	-	Weight per volume
w/w	-	Weight per weight
nm	-	Nanometer
µmol	-	Micro mol
µl	-	Micro liter
L	-	Liter
mg	-	Milligram
min	-	Minutes
mM	-	Milimolar
OD_{540}	-	Optical density at 540nm
U/ml	-	Unit of enzyme per mililiter
^{o}C	-	Degree Celcius
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

System optimization is important in any chemical reaction. The optimization process is important because it can reduce time to design or develop new products and processes. Furthermore, by using optimization system, it can develop high performance of existing processes and improve consistency and production of products. It also can reach target product and process robustness through optimization process (Montgomery, 2001).

When too many factors have an effect on the result of a process, there are several ways to optimize the process. Usually, researchers have varies one factor, while keeping the others constant and observed the factor's impact on the process. Then, the first parameter is set constant, while a second parameter is varied. This iterative step is repeated until all parameters have been optimized (Nicosia & Sciacca, 2008). The significant disadvantage of this method is that the process can be "trapped" in a local

optimum condition and the researcher cannot determine the overall optimum condition. There always have a better way to optimize parameters. One of it is through testing an organized, statistically-derived matrix of variables that ensures that the full parameter space is sampled which is the concept has been used in design of experiment (DOE) method. Before the introduction of design of experiment, the conventional methods that have been used in industry to optimize any chemical reaction commonly consume more time. Thus, the application of statistical experimental design is very important to optimize the chemical reaction effectively and accurately without consuming too much time (Montgomery, 2001).

Recently, research has been focused on the use of starch-converting enzymes in the production of glucose. The uses of starchy biomass as an industrial raw material bring commercial success of amylases, which have potential industrial applications due to the ability to hydrolyze starch to glucose. This offers a good resource in many fermentation processes (Kunamneni, 2005). Usually in optimization of enzymatic reaction process, conventional method is applied by changing one variable while maintaining all others. This method is time consuming. Experimental factorial design and response surface methodology can be applied to overcome this problem.

The main purpose of this work is to explore the potential of design of experiment to identify and optimize parameter for enzymatic reaction. In this research, DOE is used as a tool to optimize enzymatic reaction in order to obtain optimum condition for amylase activity.

1.2 Problem Statement

The application of conventional method that employ screening using one factor at a time in optimization of enzymatic reaction, result the outcome is not accurate and time consuming. Thus, the application of statistical experimental design to optimize the enzymatic reaction can overcome this problem effectively without consuming too much time. The applications of design of experiment in identify and optimize parameter for enzymatic reaction is more efficient, less time consuming, high yield, and cost-effective. Moreover, the wide application of amylase as starch-converting enzymes in the production of glucose can give lots advantages to the starch industry.

1.3 Research Objective

The objective of this research is to optimize parameter for enzymatic reaction for enzyme amylase by using design of experiment (DOE).

1.4 Scope of Study

In order to achieve the objective of the research study, several scopes have been identified:

1. To identify significant factor that affecting amylase reaction via design of experiment (DOE).
2. To optimize significant factor that affecting amylase reaction through design of experiment (DOE).

CHAPTER 2

LITERATURE REVIEW

2.1 History

Enzyme is one of the most interesting and important substances found in nature. The studies of enzyme were already performed in 1835 by the Swedish chemist Jon Jakob Berzelius. Jakob Berzelius classified their chemical as action catalytic. However, in 1926, the first pure form of enzyme was discovered by James B. Sumner of Cornell University. Sumner was able to separate and crystallize the enzyme urease from the jack bean. In 1947, John H. Northrop, Wendell M. Stanley and James B. Sumner have discovered complex procedure for isolate pepsin. The technique devised by Northrop and Stanley has been used to crystallize several enzymes (Holum, 1968).

2.2 Enzyme

The enzyme industry as we know it today is the consequence of a speedy progress seen primarily over the past four decades due to the evolution of modern biotechnology (Kirk *et al*., 2002). Most of biochemical reactions that carry on living processes would occur at high time-consuming rates without enzymes (Aehle, 2004). As a catalyst, enzyme modify the rate of reaction by provide an alternative pathway that requires less energy compare to non catalyzed reaction (McKee, 2003). The uses of enzyme as a catalyst have encouraged all researchers to find method to optimize the enzymatic reaction. There are several methods to optimize enzymatic reaction; one of the methods is by using mathematical approach. By using mathematical approach, the optimization can be done more precisely and accurately without consuming too much time (Montgomery, 2001). Their usage as a catalyst made it very important in manufacturing sectors and chemical industry. Enzymes are proteins produced by all living organisms and like all proteins, they consist of amino acids. It is also one part of group of macromolecules. Enzymes are protein and as such are amenable to structural analysis by the methods of protein chemistry, molecular biology, and molecular biophysics (Aehle, 2004). As a catalyst, they assist a chemical reaction take place rapidly and efficiently. It working by lowering the activation energy thus increases the rate of reaction. Without enzymes, some of the reactions would either happen very slowly or not occur at all (McKee, 2003). Therefore, addition of small quantity of enzyme can result a big change.

2.3 Properties of Enzymes

Enzymes are highly specific for only one particular reaction (Munksgaard, 2006). For example, enzyme maltase can breakdown only maltose and not any other disaccharide or carbohydrate. This characterization made enzyme only can perform one specific job with very few side effects (McKee, 2003). The byproduct that normally produced from chemical reaction are rarely formed through enzymatic reaction. So, it makes the process simpler and cost-effective without further treatment to separate the byproducts. The other properties of enzyme is its sensitivity to temperature. The enzyme activities depend on the temperature. Commonly, the ideal temperature for enzymatic reaction is in the range of 40°C to 60°C (Aehle, 2004). Enzyme also works well under its optimum pH. When the value of pH is less or more than optimum value, the enzyme activities will also decrease. Usually for the most enzymes, the pH optimums are between ranges from 5 to 7 (Aehle, 2004).

2.4 Factors Affecting Enzymatic Reaction

There are a number of factors that affect enzymatic reaction. For example, temperature, enzyme concentration, substrate concentration, pH value, inhibitor and others. Factors that affect enzymatic reaction must be studied because each factor gives different effect to enzymatic reaction. Some of the factors will increase enzymatic reaction as the value is increasing, but some of the factors will decrease the enzymatic reaction. The better understanding on how these factors affect enzymatic reaction enable this optimization process is conducted effectively. Besides, information of fundamental enzymatic reaction theory is important in enzyme studies to decide on a method for enzyme analysis.

2.4.1 Temperature Effects

Enzyme activity is at their maximum rate when the surrounding is in optimum value. When the temperature is too high, most enzymes become denatured. Commonly, enzyme will deactivated when temperature exceeds 40°C thus enzymatic reaction will decrease (Abusham *et al.*, 2009). There is case that the optimum temperature for enzymatic reaction is 80°C known as thermostable enzyme. Thermostable enzymes that isolated mainly from thermophilic organisms usually can endure up until 80°C (Haki & Rakshit, 2003). Figure 2.1 illustrates the common profile of temperature effect on enzyme activity.

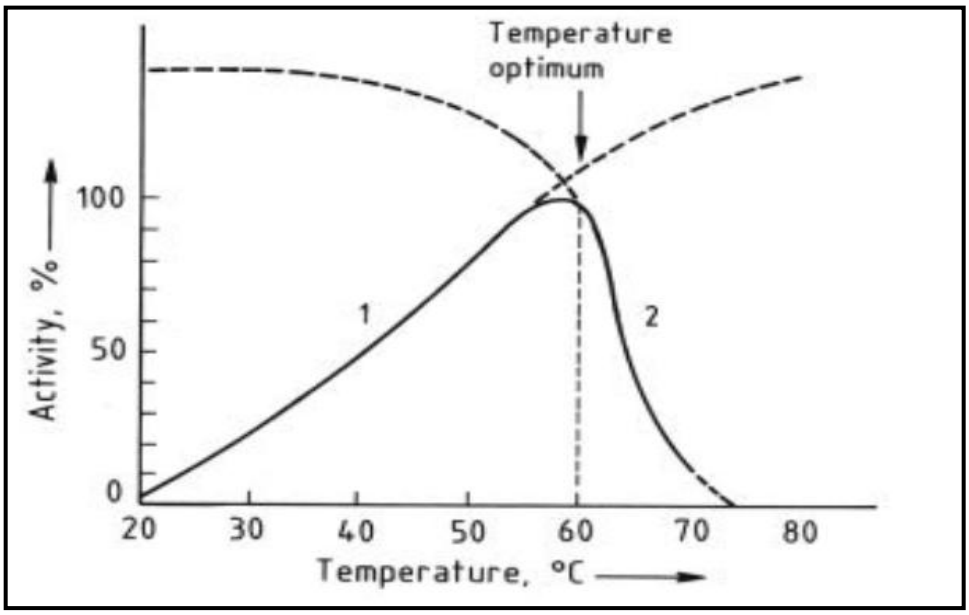

Figure 2.1: Effect of temperature to enzymatic reaction (Aehle, 2004).

2.4.2 Effects of pH

Every enzyme activity will have their specific optimum pH. At optimum pH, the enzymatic reaction is at the maximum value. To optimize the enzymatic reaction, the determination of optimum pH is very important. Below or above the optimum pH, the enzymatic reaction will decrease. The optimum values depend not only on pH but also on ionic strength and type of buffer (Aehle, 2004). Changes in pH will modify the ionic charge of the acidic and basic groups that help to maintain the specific shape of the enzyme thus the structure of enzyme will change (McKee, 2003). Figure 2.2 shows the effect of pH to enzymatic reaction on different types of enzyme.

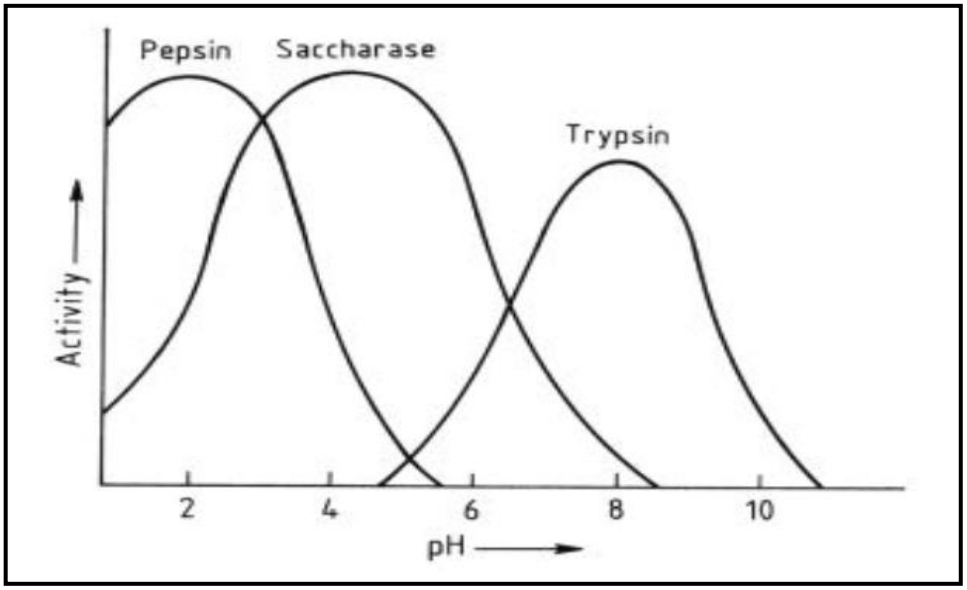

Figure 2.2: Effect of pH to enzymatic reaction (Aehle, 2004).

2.4.3 Effects of Enzyme Concentration

Rate of reaction is proportional to the enzyme concentration. When the concentration of enzymes increase, the percentages for formation of enzyme-substrate complex will increase thus the probabilities for reaction to occur will be much higher. Substrate amount must be present in excess amount to ensure the enzymatic reaction is independent to substrate concentration. Based on Figure 2.3, the reactions are said to be the zero order because the product concentration formed is proportional with time. The enzymatic reaction only affected by amount of enzyme. As the amount of enzyme increase, the rate of enzymatic reaction increase because more enzyme-substrate complex will be formed that increase the rate of reaction (Worthington, 1972). Figure 2.3 below shows the effect of amount of enzyme to product concentration.

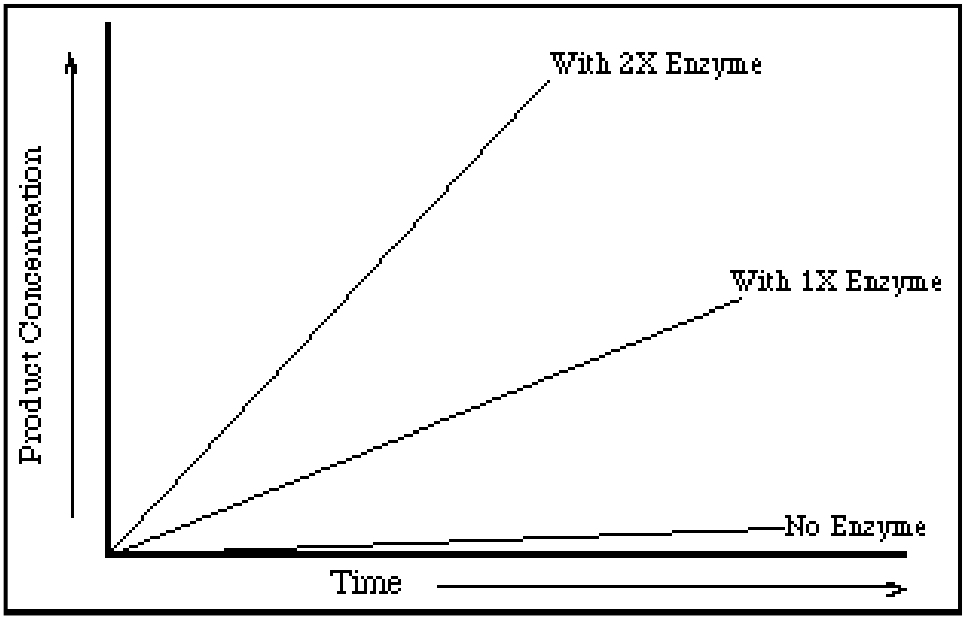

Figure 2.3: Effect of amount of enzyme to product concentration. As the amount of enzyme increase rate of reaction also increase (Worthington, 1972).

As shown in Figure 2.4, initially the curve from A to B represents zero-order reaction due to the rate of observed activity is constant with time since substrate concentration is high. The rate of reaction then slowly decreases as shown at curve B to C as substrate is finished and the enzyme's active sites are not saturated. Figure 2.4 shows the effect of enzyme activity to enzyme activity.

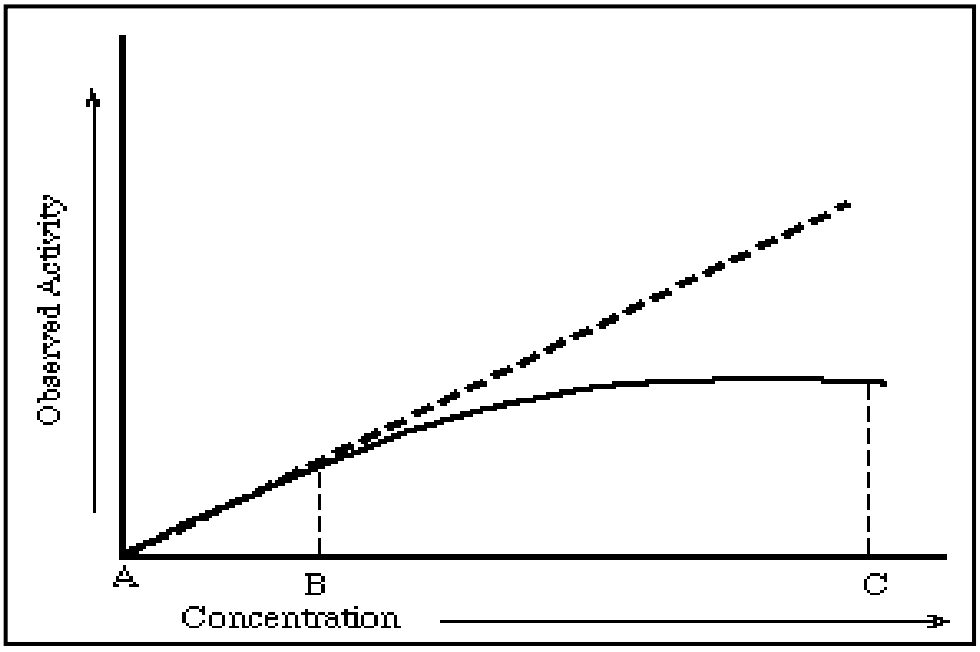

Figure 2.4: Effect of enzyme concentration to enzyme activity (Worthington, 1972).

2.4.4 Effect of Substrate Concentration

When the maximum rate of enzymatic reaction had been reached, it is indicate that the entire available enzyme has been converted to ES, which is the enzyme substrate complex (Worthington, 1972). After this point, the increases in substrate concentration

will not increase the rate of enzymatic reaction because there is no available enzyme to form an enzyme substrate complex. The effect of substrate concentration to enzymatic reaction is illustrates in Figure 2.5.

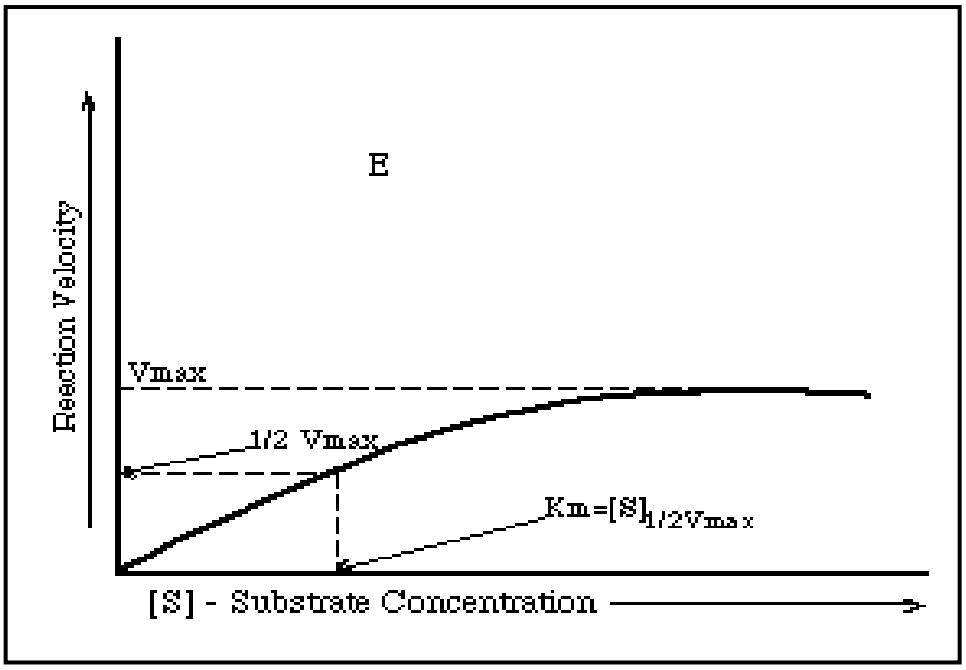

Figure 2.5: Effect of substrate concentration to reaction velocity. Km is defined as the substrate concentration at 1/2 the maximum velocity, V_{max} is defined as maximum velocity of enzymatic reaction.

2.6 Design of Experiment

Statistically a design experiment is a great tool for increasing the effectiveness of experimentation (Tye, 2004). Inclusion of duplicate test conditions allows the judgment of random, experimental variation. Statistical analysis of information obtain from the experiment clearly creates the relationship connecting the measured response and the process parameters (Altekar *et al.*, 2006). The methods for determining how to increase productivity and improve quality are evolving. Design of experiment (DOE) is one of the methods that is used to increase productivity and quality efficiently. In order to increase the productivity and quality, the optimization of any production need to be explored. The aim in this methodology is to optimize the response so that the production yield will be increase for an experiment. The objective in design of experiment is to reduce time to design or develop new processes, develop high performance of existing processes, improve consistency, and reach target product and process robustness.

In design of experiment, there were basic principles that need to be followed in order to attain the main objective. There are three basic principles in experimental design which is replication, randomization, and blocking (Montgomery, 2001). First basic principle is replication which means a repetition. In replication, the experimenters are able to obtain a more precise estimate of this effect. The second basic principle is randomization. It is the cornerstone fundamental of the use of statistical methods in experimental design. Through randomization, both allocation experimental materials and the order of experiment are to be done in random ways. The last one is blocking which is a design technique used to improve the precision with which comparisons among the factors of interest are made. Generally, blocking is used to eliminate and reduce variables that are not influencing the experimental response directly. All three principles are the most important part in any experimental design (Montgomery, 2001).

2.6.1 Advantages of Design of Experiment

Design of experiment has several advantages in several aspects. The application of design of experiment is extensively used in various field because the advantages of design of experiment. The advantages of design of experiment are:

1. DOE can be used to find solutions in all manufacturing elements including product design, manufacturing technology, handling and support system.
2. DOE can be utilized as a decision tool to find the best setting for those design factors so that optimum performance can be achieved.
3. DOE also common to see engineers in manufacturing using trial and error approach to find the best setting of a system to optimize performance.
4. Reduce time consuming, cost, and design process.
5. Increase yield, stability and percentage to obtain target product.

2.6.2 Type of Design of Experiment

Generally, there are three main types in design of experiment since four eras of modern development of statistical design, which are factorial design, Taguchi method, and response surface methodology. Factorial design is the most efficient method among the others. It is commonly employed in manufacturing applications to analytically and efficiently discover the set of input variables, or parameters on responses such as yield (SAS institute Inc, 2005). A properly designed experiment can decrease the number of experimental runs that would otherwise be needed if this approach or random sampling

is applied. Almost all experiments involve the study of the effect of two or more parameters.

The second type is Taguchi method. Taguchi has developed a method based on Orthogonal Array experiments which reduced the quantity of variance for the experiment with optimum settings of control parameters. The main objective is to reduce the variability of value characteristics around a target (SAS institute Inc, 2005). Third type is response surface methodology. Response surface methodology or RSM is a set of statistical and mathematical procedure that are functional in modeling and analysis of problem in which response of interest is influenced by several parameters that already been identified before the experiment. This method is developed by Box and Wilson in 1951.

2.6.3 Application of Design of Experiment

Recently, there is several researches that focused on the optimization by using design of experiment. Design of experiment has been used in several fields for example in aerospace engineering, computer industries, micro component production and others. This shows the wide application of design of experiment as a tool to optimize process. Table 2.1 summarized the application of design of experiment involved enzymatic reaction.

Table 2.1: Parameter optimization of enzymatic reactions references from selected journals involved enzymatic reaction.

Enzyme	**Type of Design of Experiment**	**Optimized Parameters**	**Reference**
Amylase	Response Surface Methodology (RSM)	pH (7.1) , Temperature (57.5°C), Time (25 min)	(Agrawal *et al*., 2004)
Lipase	Response Surface Methodology (RSM), Central Composite Design (five-level-four-factor)	Reaction time (349.2 min) , temperature (52.9°C) , and enzyme amount (37.8%w/w)	(Twu, Shih, Yen, Ling, & Shieh, 2005)
Immobilized lipase	Response Surface Methodology (RSM with five-level-four-factors)	Reaction temperature (43.06°C), reaction time (164.25 min), enzyme amount (7.47%) and substrate molar ratio (3.98:1).	(Jeong & Park, 2006)
Cyclodextrin Glucanotransferase	Response surface methodology (RSM), 2^4 Factorial designs, 2^2 Central composite designs.	Amount of yeast extract (1.89%) & sago starch (1.48%)	(Mahat *et al*., 2004)

CHAPTER 3

METHODOLOGY

3.1 Material

All chemicals used in this research were provided by Laboratory of Faculty of Chemical and Natural Resources Engineering (FKKSA), UMP. Most chemicals were bought from Merck, Sigma and Fluka.

3.2 Amylase Assay

The determination of amylase activity was carried out based on Agrawal *et al.*, (2004). Amylase enzyme was assayed by adding 0.1 ml of amylase enzyme to 5 mg of soluble starch that have been diluted in 900 µl of 100 mM potassium phosphate buffer of pH 6.0. Then the solution was incubated in water bath at 50 °C for 15 minutes. One

milliliter of 3, 5-dinitrosalicylic acid was added to stop the reaction. The solution mixture was finally heated up to develop color and terminate the reaction simultaneously. The amount of reducing sugars was measured at 540 nm by the method of Miller (1959) with slight modification. Under the standard assay conditions, one unit of enzyme activity is defined as the amount of enzyme that releases 1 µmol of reducing sugars per min (Agrawal *et al*., 2004).

3.3 Preparation of Standard Curve

Glucose stock solution was prepared by dissolved 0.3g of glucose into 100ml of distilled water, giving a stock concentration of 3 g/l. The stock solution then was diluted to series of dilution by using distilled water. The series concentrations were 2.5 g/l, 2.0 g/l, 1.5 g/l and 1.0 g/l. glucose. The solution was identified as non enzyme solution. Based on amylase assay, the solutions were added with 5 mg soluble starch that already been diluted in 900µl of 100mM of potassium phosphate buffer of pH 6.0 followed by incubation of reaction mixture at 50°C for 15 minutes in water bath. The solutions were mixed with 1ml of DNS reagent to stop the reaction. Then the absorption was measured at 540 nm and the graph OD_{540} versus glucose concentration was plotted.

3.4 Factorial Screening

Based on Design Expert Software Version 7.1.6, the matrix design of 2^4 full factorial design was developed. The matrix design was used to show the statistical significant parameter that affect enzymatic reaction. The factors were pH, temperature, enzyme concentration and substrate concentration. To determine the significant parameter that affecting enzymatic reaction of amylase, a total of 32 experiments were conducted. The ranges of parameters were determined based on the literature. Table 3.1 shows the initial data used in 24 full factorial design.

Table 3.1: Initial data used in 2^4 full factorial design.

Factor	Unit	Actual value		Coded value	
		Low	High	Low	High
pH		4	9	-1	1
Temperature	°C	40	80	-1	1
Enzyme conc	U/ml	5	15	-1	1
Substrate conc	%w/v	2	6	-1	1

Coding of the variables was done according to the following equation 3.1:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad (3.1)$$

where, x_i = coded value of the *i*th independent variable,

X_i = actual value of the *i*th independent variable,

X_0 = actual value of the *i*th independent variable at the centre point,

ΔX_i = step change.

After this step, the design expert software will give several number of run that need to be followed in order to eliminate insignificant parameter. For eliminating insignificant parameter, the design was made up from 2-level factorial design. In this step, the experiment will be done with two times of replication resulting 32 runs that needed to be performed by using table below as guidance. The table that obtained from design expert software was as shown in Table 3.2.

Table 3.2: Numbers of run with factor affecting amylase activity that obtained from design expert software.

Standard order	Run	Factor 1	Factor 2	Factor 3	Factor 4
		A:pH	B:Temperature (°C)	C:Enzyme concentration (U/ml)	D:Substrate concentration (%w/v)
1	12	4.00	40.00	5.00	2.00
2	30	4.00	40.00	5.00	2.00
3	21	9.00	40.00	5.00	2.00
4	26	9.00	40.00	5.00	2.00
5	19	4.00	80.00	5.00	2.00
6	2	4.00	80.00	5.00	2.00
7	9	9.00	80.00	5.00	2.00
8	6	9.00	80.00	5.00	2.00
9	7	4.00	40.00	15.00	2.00
10	18	4.00	40.00	15.00	2.00
11	20	9.00	40.00	15.00	2.00
12	24	9.00	40.00	15.00	2.00
13	16	4.00	80.00	15.00	2.00
14	31	4.00	80.00	15.00	2.00

15	3	9.00	80.00	15.00	2.00
16	17	9.00	80.00	15.00	2.00
17	1	4.00	40.00	5.00	6.00
18	5	4.00	40.00	5.00	6.00
19	10	9.00	40.00	5.00	6.00
20	14	9.00	40.00	5.00	6.00
21	25	4.00	80.00	5.00	6.00
22	11	4.00	80.00	5.00	6.00
23	4	9.00	80.00	5.00	6.00
24	13	9.00	80.00	5.00	6.00
25	29	4.00	40.00	15.00	6.00
26	8	4.00	40.00	15.00	6.00
27	15	9.00	40.00	15.00	6.00
28	27	9.00	40.00	15.00	6.00
29	23	4.00	80.00	15.00	6.00
30	28	4.00	80.00	15.00	6.00
31	22	9.00	80.00	15.00	6.00
32	32	9.00	80.00	15.00	6.00

Amount of glucose produced was considered as a dependent variable whiles parameter such as pH of buffer, temperature, enzyme concentration and substrate concentration were considered as independent variables (Agrawal *et al*, 2004). The time reaction and other parameters were maintained constant. Factorial design can eliminate the insignificant parameter that not effectively effecting enzymatic reaction by performing analysis of variance (ANOVA). It will perform by design expert software. After do this step, the insignificant parameter will be eliminated.

3.5 Optimization of Enzymatic Reaction by using Response Surface Methodology with Central Composite Design

All significant parameters that obtained in the screening stage were used to be optimized by response surface methodology with central composite design. The insignificant factors were maintained at the minimum point. The time reaction and other parameters were maintained constant. The value of alpha was set at 1.681791, which determined the location of the star points in a central composite design. By implementing two replicates in the design, the matrix was produced by the software as shown in Table 3.3.

Table 3.3: The matrix of response surface methodology with central composite design.

Std	Run	Factor 1	Factor 2	Factor 3
		A:pH	B:Temperature °C	C:Substrate Concentration %w/v
1	17	4.00	40.00	2.00
2	18	4.00	40.00	2.00
3	22	9.00	40.00	2.00
4	23	9.00	40.00	2.00
5	5	4.00	80.00	2.00
6	25	4.00	80.00	2.00
7	7	9.00	80.00	2.00
8	15	9.00	80.00	2.00
9	2	4.00	40.00	6.00
10	26	4.00	40.00	6.00
11	1	9.00	40.00	6.00
12	4	9.00	40.00	6.00
13	9	4.00	80.00	6.00
14	34	4.00	80.00	6.00
15	16	9.00	80.00	6.00
16	30	9.00	80.00	6.00
17	20	2.30	60.00	4.00
18	12	2.30	60.00	4.00
19	32	10.70	60.00	4.00
20	11	10.70	60.00	4.00
21	6	6.50	26.36	4.00
22	14	6.50	26.36	4.00
23	29	6.50	93.64	4.00
24	21	6.50	93.64	4.00
25	24	6.50	60.00	0.64
26	10	6.50	60.00	0.64
27	27	6.50	60.00	7.36
28	8	6.50	60.00	7.36
29	13	6.50	60.00	4.00
30	28	6.50	60.00	4.00
31	33	6.50	60.00	4.00
32	3	6.50	60.00	4.00
33	19	6.50	60.00	4.00
34	31	6.50	60.00	4.00

CHAPTER 4

RESULT AND DISCUSSION

4.1 Two - level Full Factorial Design

The reported optimum value in literature for pH, temperature, enzyme and substrate concentrations were 6.0, 50 ℃, 12 U/ml and 4.5% (w/v) (Baskar *et al*., 2008; Agrawal *et al*.,2004). Hence the range of pH, temperature, enzyme concentration and substrate concentration were 5 – 8, 40°C – 80 ℃, 5 U/ml – 15U/ml and 2% – 6% (w/v), respectively.

In order to obtain the significant parameter that have high effect on enzymatic hydrolysis, the two-level factorial design was performed. Full factorial design allowed us to estimate linear and two factor interaction effect of the factor and can be used to estimate the effects of all interactions. Factorial design offer a reduction in number of experiment without losing a lot of information.

Table 4.1 represents the results of experiment that obtained from the experimental works. The actual value was the value that obtained from the experimentation work and the predicted value was the value that predict by the software. The significant variables that highly affected the enzyme activity were determined through analysis of variance (ANOVA).

Table 4.1: Experimental design of 2^4 full factorial design with experimental and predicted values.

Std	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	
		A:pH	B: Temperature (°C)	C: Enzyme concentration (U/ml)	D: Substrate concentration (%w/v)	Enzyme activity (U/ml) Actual	Enzyme activity (U/ml) Predicted
1	12	4.00	40.00	5.00	2.00	2.09854	2.14
2	30	4.00	40.00	5.00	2.00	2.16921	2.14
3	21	9.00	40.00	5.00	2.00	2.1378	2.13
4	26	9.00	40.00	5.00	2.00	2.11817	2.13
5	19	4.00	80.00	5.00	2.00	2.2546	2.21
6	2	4.00	80.00	5.00	2.00	2.18001	2.21
7	9	9.00	80.00	5.00	2.00	2.2811	2.28
8	6	9.00	80.00	5.00	2.00	2.28111	2.28
9	7	4.00	40.00	15.00	2.00	2.09854	2.10
10	18	4.00	40.00	15.00	2.00	2.08087	2.10
11	20	9.00	40.00	15.00	2.00	2.08087	2.08
12	24	9.00	40.00	15.00	2.00	2.08087	2.08
13	16	4.00	80.00	15.00	2.00	2.2546	2.25
14	31	4.00	80.00	15.00	2.00	2.2546	2.25
15	3	9.00	80.00	15.00	2.00	2.24233	2.26
16	17	9.00	80.00	15.00	2.00	2.26785	2.26
17	1	4.00	40.00	5.00	6.00	2.20356	2.19
18	5	4.00	40.00	5.00	6.00	2.20356	2.19
19	10	9.00	40.00	5.00	6.00	2.1378	2.15
20	14	9.00	40.00	5.00	6.00	2.15841	2.15
21	25	4.00	80.00	5.00	6.00	2.23105	2.25
22	11	4.00	80.00	5.00	6.00	2.2546	2.25
23	4	9.00	80.00	5.00	6.00	2.2811	2.30
24	13	9.00	80.00	5.00	6.00	2.31055	2.30

25	29	4.00	40.00	15.00	6.00	2.1378	2.14
26	8	4.00	40.00	15.00	6.00	2.15841	2.14
27	15	9.00	40.00	15.00	6.00	2.18001	2.18
28	27	9.00	40.00	15.00	6.00	2.18001	2.18
29	23	4.00	80.00	15.00	6.00	2.29583	2.28
30	28	4.00	80.00	15.00	6.00	2.2546	2.28
31	22	9.00	80.00	15.00	6.00	2.37631	2.36
32	32	9.00	80.00	15.00	6.00	2.34343	2.36

Half-normal plot was used to obtain the significant parameter that affecting amylase activity. The plot was obtained when the experimental data were fitted in the Design Expert software. Only those effects that are clearly off the line were chosen as significant effects. From the Figure 4.1, there were eight points that fell off the line indicated that the factor and interaction between those factor were significant to model.

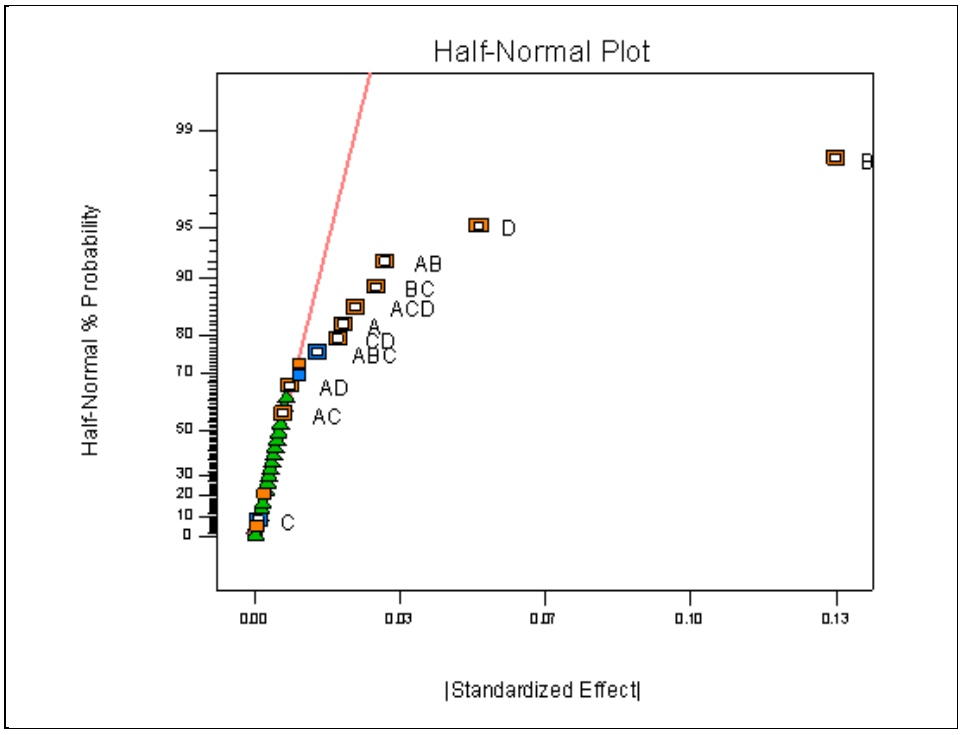

Figure 4.1: Half-Normal plot. There were eight points that fell far from line indicated the factors were significant.

Analysis of variance (ANOVA) and regression analysis for the enzymatic reaction of α-amylase is shown in Table 4.2. Determination of coefficient (R^2) and correlation coefficient (R) indicates the precision of a model. Determination coefficient (R^2) implied that the independent variables tested were attributed by the sample variation of 94.93% for α-amylase activity. The Model F-value which was 34.02 indicated the model was significant. Prob > F less than 0.05 indicates model terms have a significant effect on the response.

Factor A, B, and D have value of Prob > F less than 0.1, which indicated that factor A, B, and D (pH, temperature and substrate concentration, respectively) were higly significant to the enzymatic reaction while enzyme concentration did not give high effect to the enzymatic reaction of α-amylase. The Lack of Fit F-value of 0.81 shows the Lack of Fit is not significant relative to the pure error. There is a 53.61% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit.

The equation model generated based on regression model analysis is as follows:

$$Y = 2.21 + 0.010A + 0.067B - 4.448E^{-4}C + 0.026D + 0.015\,AB + 3.297E^{-3}\,AC + 4.034E^{-3}AD + 0.014BC + 9.555E^{-3}CD - 7.254E^{-3}ABC + 0.012ACD \quad (4\ 1)$$

Where Y was enzyme activity (U/ml) and A, B, C, D, AB, AC, AD, BC, CD, ABC and ACD were pH, temperature (°C), enzyme concentration (U/ml), substrate concentration (% w/v), pH-temperature interaction, pH-enzyme concentration interaction, pH substrate concentration interaction, temperature-enzyme concentration interaction, enzyme-substrate concentration interaction, pH-temperature-enzyme concentration interaction and pH-enzyme-substrate concentration interaction.

Table 4.2: Analysis of variance for selected factorial model.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	P-value Prob > F
Model	0.19	11	0.017	34.02	< 0.0001
A-pH	$3.349x10^{-3}$	1	$3.349 x10^{-3}$	6.57	0.0186
B-Temperature	0.14	1	0.14	280.42	< 0.0001
C-Enzyme concentration	$6.332 x10^{-6}$	1	$6.332 x10^{-6}$	0.012	0.9124
D-Substrate concentration	0.021	1	0.021	41.80	< 0.0001
AB	$7.214 x10^{-3}$	1	$7.214 x10^{-3}$	14.14	0.0012
AC	$3.479 x10^{-4}$	1	$3.479 x10^{-4}$	0.68	0.4186
AD	$5.206 x10^{-4}$	1	$5.206 x10^{-4}$	1.02	0.3244
BC	$6.192 x10^{-3}$	1	$6.192 x10^{-3}$	12.14	0.0023
CD	$2.921 x10^{-3}$	1	$2.921 x10^{-3}$	5.73	0.0266
ABC	$1.684 x10^{-3}$	1	$1.684 x10^{-3}$	3.30	0.0842
ACD	$4.291 x10^{-3}$	1	$4.291 x10^{-3}$	8.41	0.0088
Residual	0.010	20	$5.100 x10^{-4}$		
Lack of Fit	$1.720 x10^{-3}$	4	$4.300 x10^{-4}$	0.81	0.5361
Pure Error	$8.480 x10^{-3}$	16	$5.300 x10^{-4}$		
Cor Total	0.20	31			

The large values of coefficient of model indicate that it had more significant effects on the amylase activity (Mahat *et al*., 2004). Based on Equation 4.1, the largest coefficient value of 0.067 indicated that temperature was the most significant parameter that affecting amylase activity. In addition, the variable with positive fitted constant has an enhancer influence towards amylase activity than the one with negative coefficient, which had inhibitory influence.

4.1.1 Model Confirmation

Additional diagnostic plots need to be examined in order to certify the model. There were three plots that need to be discussed further which were normal plot of residuals, residuals versus predicted values, and predicted versus actual plot.

Normal probability plot was examined to check the normality of this model as illustrated in Figure 4.2. It was clear that there were two points that fell far from the line. It shows an abnormality in the plot. The plot needs to be normally distributed in order for the model to be valid. Furthermore, the shape of the plot fairly exhibited an "S" shape indicating an abnormality in the plot. It was suggested that a transformation of the response may provide a better analysis (Haaland, 1989).

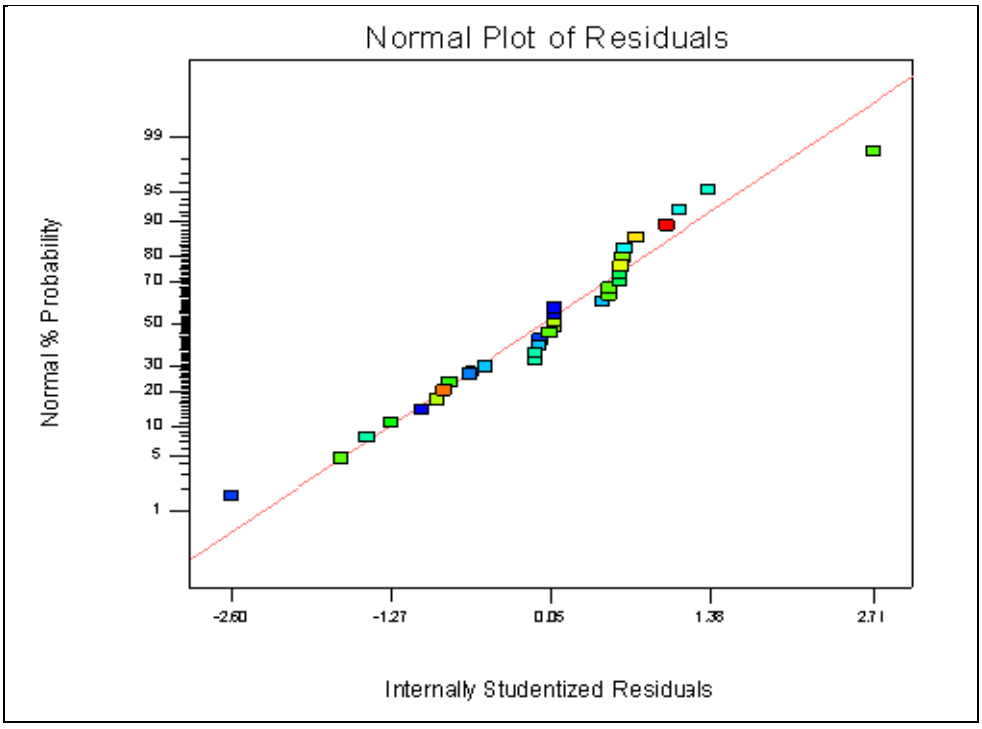

Figure 4.2: Normal plot of residual. There were two points fell far from line shows an abnormality.

Residual versus predicted plot was diagnosed to test the assumption of constant variance. The plot should be random scatter means constant range of residuals across the graph. Based on Figure 4.3, all the point lies on range between 3.00 and -3.00 indicating that the model was in the predicted range. There was also no pattern such megaphone shape, which indicates non constant variance for the residuals.

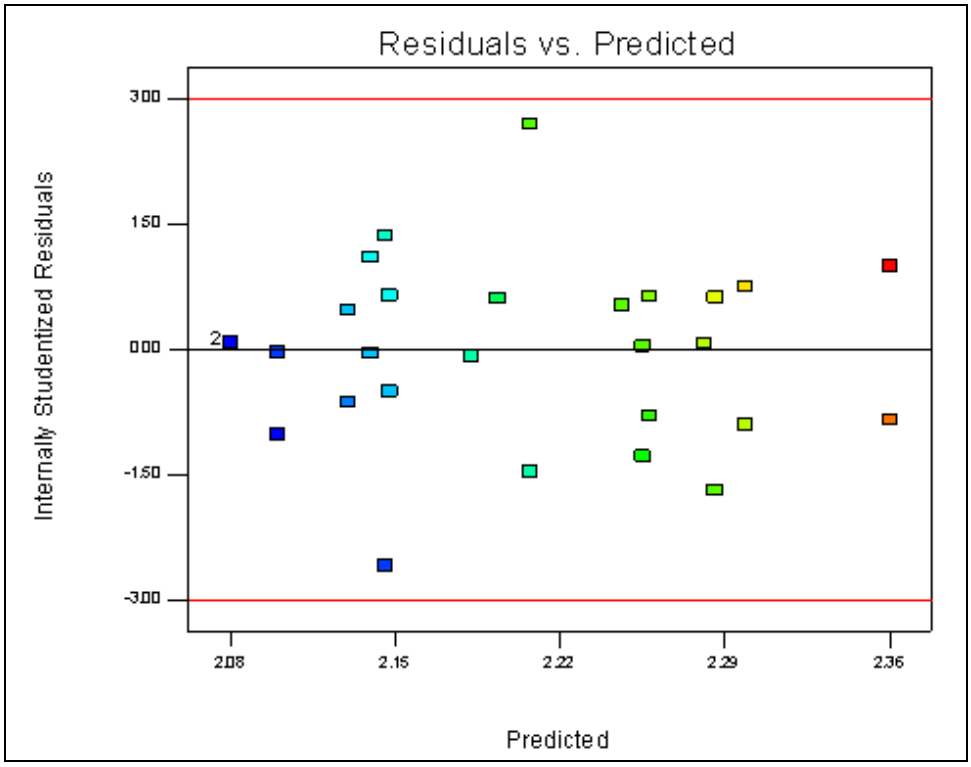

Figure 4.3: Residual versus Predicted plot. All points randomly scatter and lies on range between 3.00 and -3.00.

Another figure that needs to be diagnosed is the predicted versus actual plot as shown in Figure 4.4. A good agreement between actual and predicted values was observed as the points are dotted along the straight line.

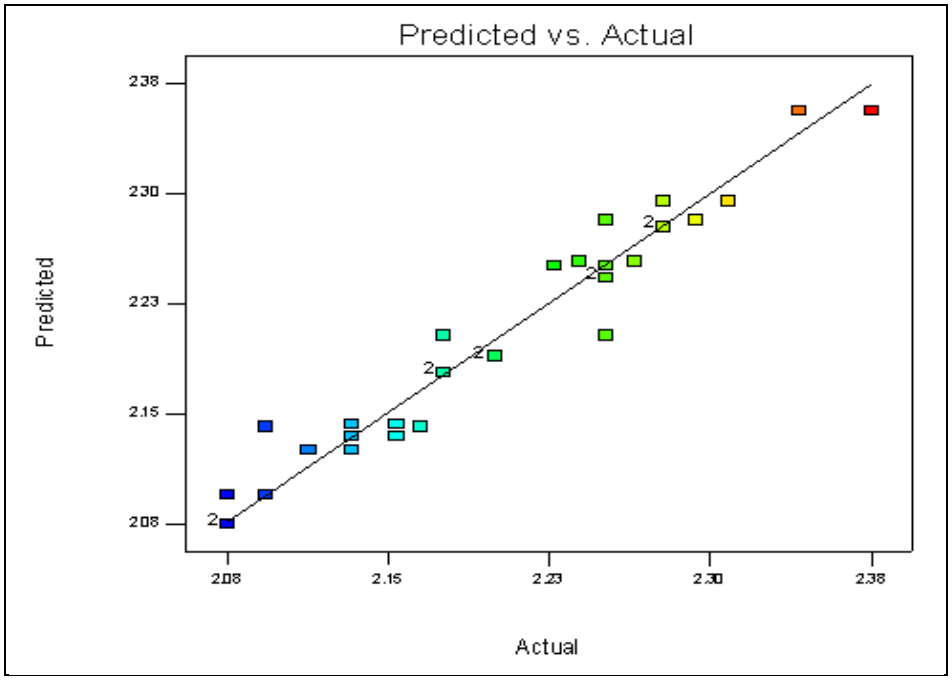

Figure 4.4: Predicted versus Actual plot.

4.2 Response Surface Methodology

Significant factors from full factorial design were further optimized using response surface methodology. After eliminating the insignificant parameter, same the range of significant parameter obtained was used in this step. The optimum condition for amylase activity can be determined by using response surface plot. Response surface plot can be obtained by employing the central composite design based on experimental data. Table 4.3 shows the results of experiment that obtained from the experimental works.

Table 4.3: Experimental design and results of the central composite design with actual and predicted values.

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	
		A:pH	B:Temperature (°C)	C:Substrate Concentration (%w/v)	Enzyme activity (U/ml) Actual	Enzyme activity (U/ml) Predicted
1	17	4.00	40.00	2.00	1.58225	1.48
2	18	4.00	40.00	2.00	1.38659	1.48
3	22	9.00	40.00	2.00	1.69709	1.70
4	23	9.00	40.00	2.00	1.97192	1.70
5	5	4.00	80.00	2.00	1.88456	1.76
6	25	4.00	80.00	2.00	1.94493	1.76
7	7	9.00	80.00	2.00	2.00038	1.88
8	15	9.00	80.00	2.00	2.01511	1.88
9	2	4.00	40.00	6.00	1.93511	2.11
10	26	4.00	40.00	6.00	2.09854	2.11
11	1	9.00	40.00	6.00	2.1378	2.18
12	4	9.00	40.00	6.00	2.00038	2.18
13	9	4.00	80.00	6.00	2.08087	2.13
14	34	4.00	80.00	6.00	1.98566	2.13
15	16	9.00	80.00	6.00	2.10933	2.10
16	30	9.00	80.00	6.00	2.15841	2.10
17	20	2.30	60.00	4.00	2.08116	2.00
18	12	2.30	60.00	4.00	2.00038	2.00
19	32	10.70	60.00	4.00	2.05211	2.16
20	11	10.70	60.00	4.00	2.07498	2.16
21	6	6.50	26.36	4.00	2.11162	2.05
22	14	6.50	26.36	4.00	2.09854	2.05
23	29	6.50	93.64	4.00	2.11817	2.21
24	21	6.50	93.64	4.00	2.08156	2.21
25	24	6.50	60.00	0.64	0.964856	1.19
26	10	6.50	60.00	0.64	0.938354	1.19
27	27	6.50	60.00	7.36	2.06939	1.91
28	8	6.50	60.00	7.36	2.10508	1.91
29	13	6.50	60.00	4.00	1.90812	1.91
30	28	6.50	60.00	4.00	1.87376	1.91
31	33	6.50	60.00	4.00	1.97192	1.91
32	3	6.50	60.00	4.00	1.92117	1.91
33	19	6.50	60.00	4.00	1.92382	1.91
34	31	6.50	60.00	4.00	1.88849	1.91

Based on the result obtained, a quadratic model for amylase activity was given by Design Expert software. Table 4.4 summarizes the ANOVA for the optimization process. Determination coefficient (R^2) implies that the independent variables tested were attributed by the sample variation of 81.86% for α-amylase activity. According to Joglekar and May (1987), the R^2 should be at least 80% for the good fit of model. The Model F-value of 12.04 indicated the model is significant.

The Lack of Fit F-value of 15.96 shows the Lack of Fit is significant relative to the pure error. Significant lack of fit is not good. There were several reasons that contribute to this situation such as, error due to data entry, problem with equipment, bad measurement during experimentation works and unknown lurking variable that appear only intermitently (Anderson and Whitcomb, 2003). However, repetition of a few experiments did not improve the fit of the model.

Table 4.4: Analysis of variance for selected response surface methodology.

Source	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	2.31	9	0.26	12.04	< 0.0001
A-pH	0.059	1	0.059	2.76	0.1096
B-Temperature	0.067	1	0.067	3.14	0.0892
C-Substrate Concentration	1.25	1	1.25	58.59	< 0.0001
AB	0.011	1	0.011	0.51	0.4818
AC	0.021	1	0.021	0.99	0.3304
BC	0.068	1	0.068	3.20	0.0864
A^2	0.079	1	0.079	3.69	0.0668
B^2	0.13	1	0.13	6.24	0.0197
C^2	0.38	1	0.38	17.67	0.0003
Residual	0.51	24	0.021		
Lack of Fit	0.41	5	0.083	15.96	< 0.0001
Pure Error	0.098	19	5.181E-003		
Cor Total	2.82	33			

The multiple regression equation that obtained form Design Expert software that shows the effect of significant parameter to amylase activity is as follows:

$$Y = 1.00417 - 0.043931\,A - 0.010657\,B + 0.51065\,C - 5.21853E^{-4}\,AB - 7.25523E^{-3}\,AC - 1.63232E^{-3}\,BC + 9.44920E^{-3}\,A^2 + 1.92113E^{-4}\,B^2 - 0.032324\,C^2 \quad (4\ 2)$$

Where Y was enzyme activity (U/ml) and A, B, C, AB, AC, BC, A^2, B^2 and C^2 were pH, temperature (°C), substrate concentration (% w/v), pH-temperature interaction, pH-substrate concentration interaction, and temperature-substrate concentration interaction. The highest conversion of glucose which produced 2.15841 U/ml of enzyme activity was recorded on standard number 16. The parameters of standard number 16 were 80^{o}C temperature, pH 9 and 6% (w/v) of substrate concentration with 5 U/ml of enzyme concentration. The lowest conversion of glucose was recorded at standard number 26 with 0.938354 U/ml enzyme activities. The parameters for standard number 26 were 60^{o}C, pH 6.5 and 7.36% (w/v) with 5 U/ml of enzyme concentration. By comparing highest conversion with lowest conversion, the interaction between parameters can be understood. For highest conversion of glucose, amylase used can endure high temperature until 80^{o}C. At high temperature, number of collision between enzyme and substrate will increase because more molecules have sufficient energy to enter the transition state (McKee, 2003). This will increase amylase reaction. More basic indicates low hydrogen ion (McKee, 2003). It has effect on enzymatic reaction which changes the ionization of active site. Moreover, substrates can also affecting amylase activity. Low concentration indicates more available active site of amylase enzyme.

Figure 4.5 shows the distribution of studentized residuals which is in linear pattern. This indicates the residual are distributed normally.

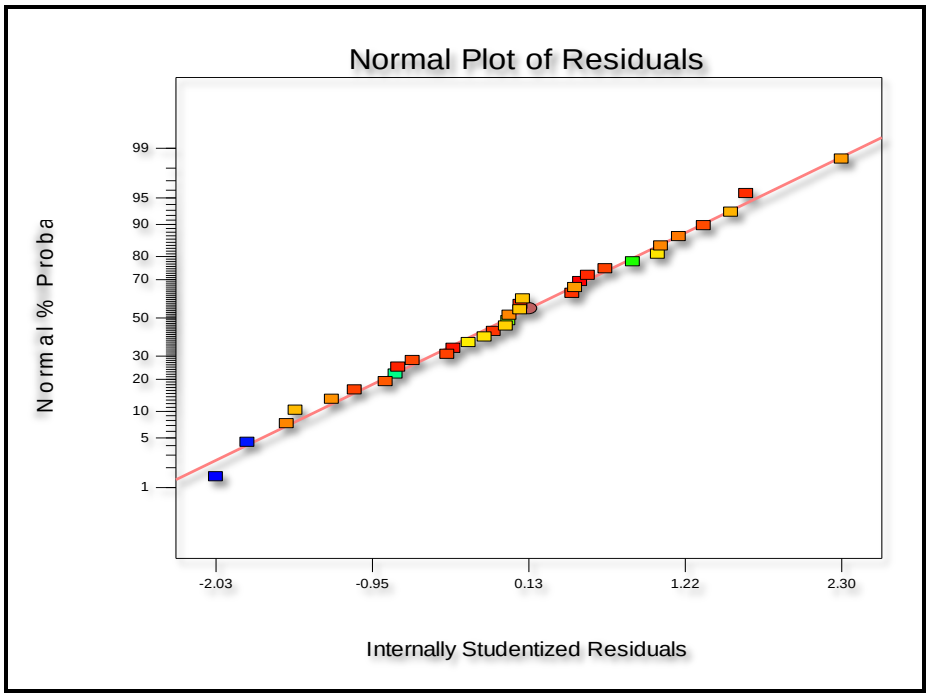

Figure 4.5: Normal plot of residual for response surface methodology.

Residual versus predicted value plot was diagnosed to test the assumption of constant variance. This plot should randomly scatter and all point should scattered inside the constant range of residual across the graph which is 3.00 to -3.00. Based on Figure 4.6, all the point not randomly scattered but still inside the constant range of residuals. There is no pattern such megaphone pattern indicates no transformation needed to improve the model.

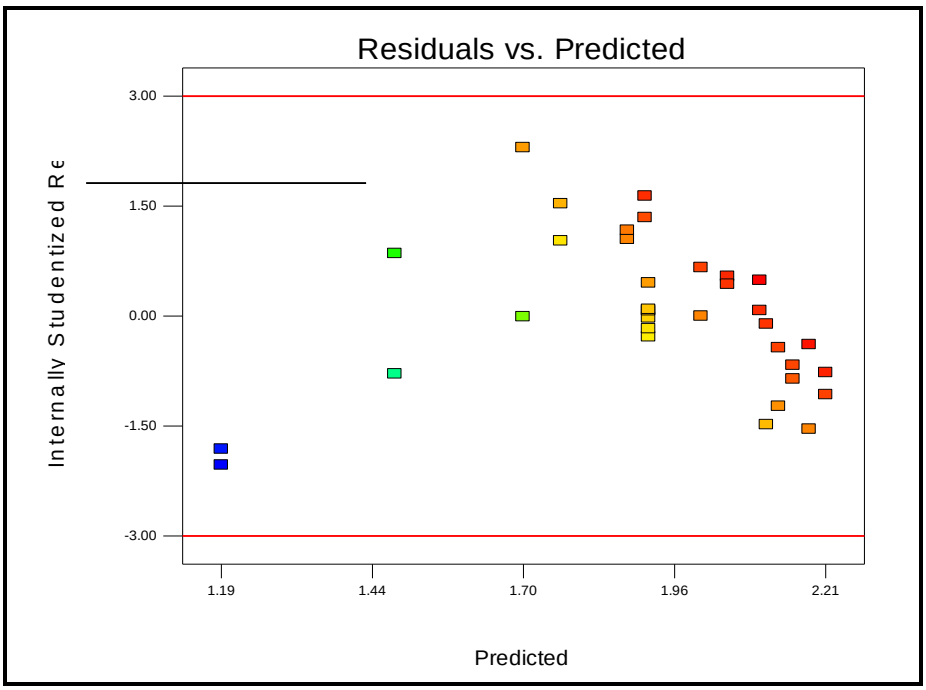

Figure 4.6: Residual versus predicted for response surface methodology.

Another figure to examine is externally studentized residuals or outlier T plot to look for outliers for example influential values, as depicted in Figure 4.7. There is no outliers occur. If there was an outlier, the repetition of experiment for that specific run needed. The point also scattered inside the constant range of residual indicates there is no need for transformation to improve the model.

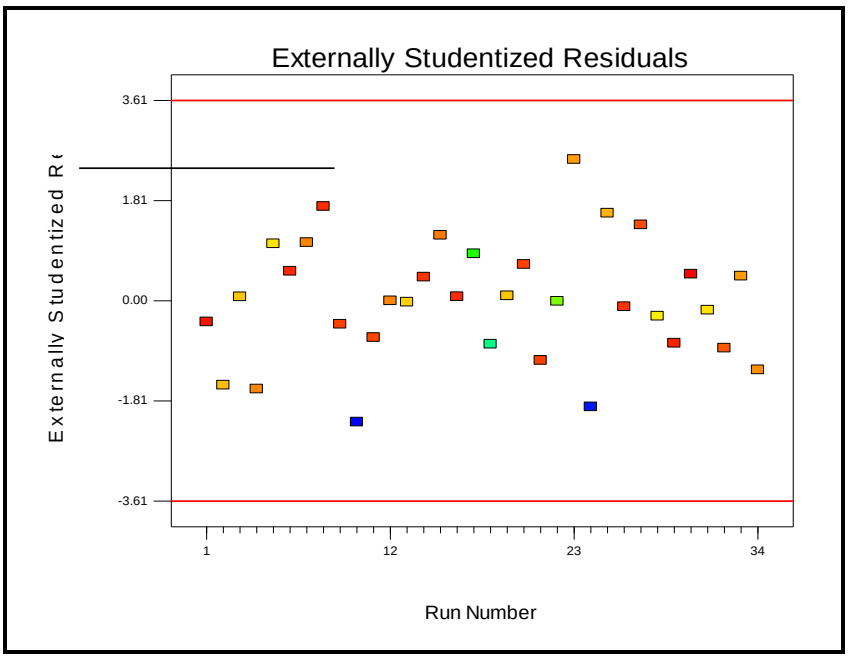

Figure 4.7: Externally studentized residuals (Outlier T).

In order to identify the temperature, pH and substrate concentration on hydrolysis of starch, a response surface plot (3D plot) that obtained from Design Expert software was investigated. Figure 4.8 shows the contour of response surface plot for effect of pH and temperature interaction to amylase activity. The red colour region indicates the maximum amylase activity. Figure 4.9 shows the response surface plot that obtained from the software. Based on Figure 4.9, the response surface plot shows the interaction between pH and temperature to the amylase activity. Temperature and pH give effect to the amylase activity, and as high temperature used, high amylase activity recorded. From the figure, the peak of the plot cannot be determined. This happen due to optimum value for amylase enzyme may be out of range used.

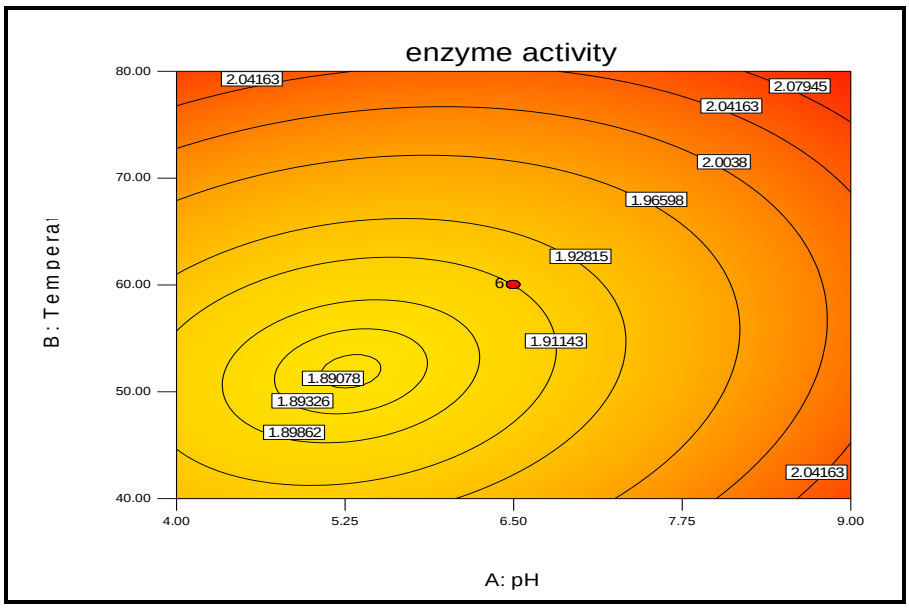

Figure 4.8: Contour plot of amylase activity from model equation – effect of temperature and pH.

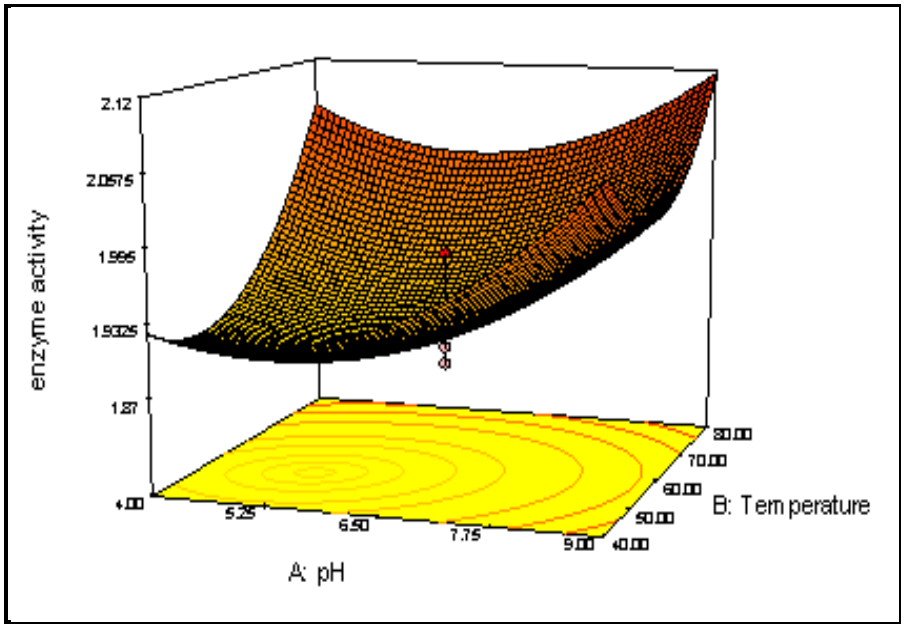

Figure 4.9: Response surface plot from model equation – effect of pH and temperature.

Figure 4.10 shows the contour of response surface plot for effect of pH and substrate concentration interaction to amylase activity and Figure 4.11 shows the response surface plot that obtained from the software. Based on Figure 4.11, substrate concentration and pH give effect to the amylase activity, and as high substrate concentration used, high amylase activity recorded. However, pH was slightly effecting on amylase activity.

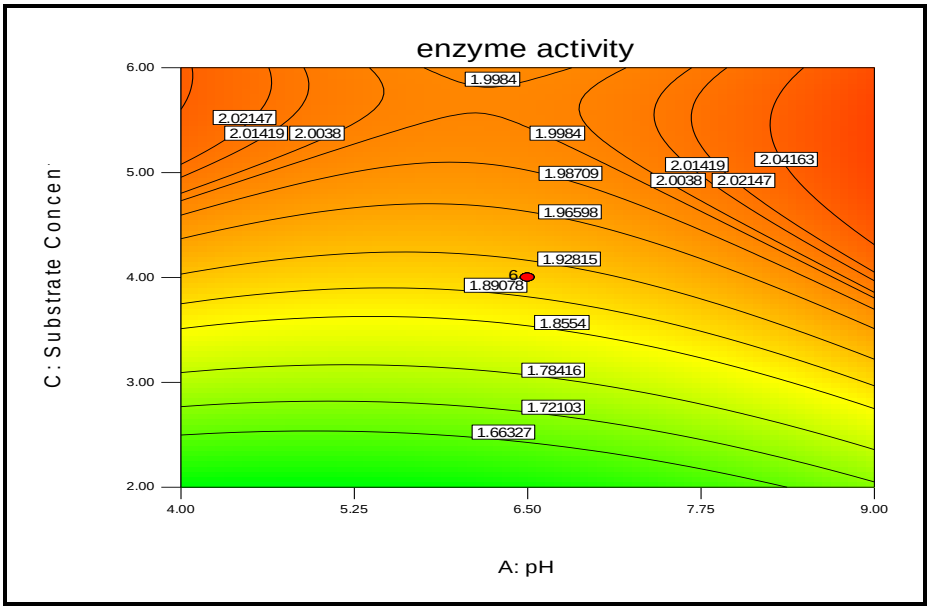

Figure 4.10: Contour plot of amylase activity from model equation – effect of substrate concentration and pH.

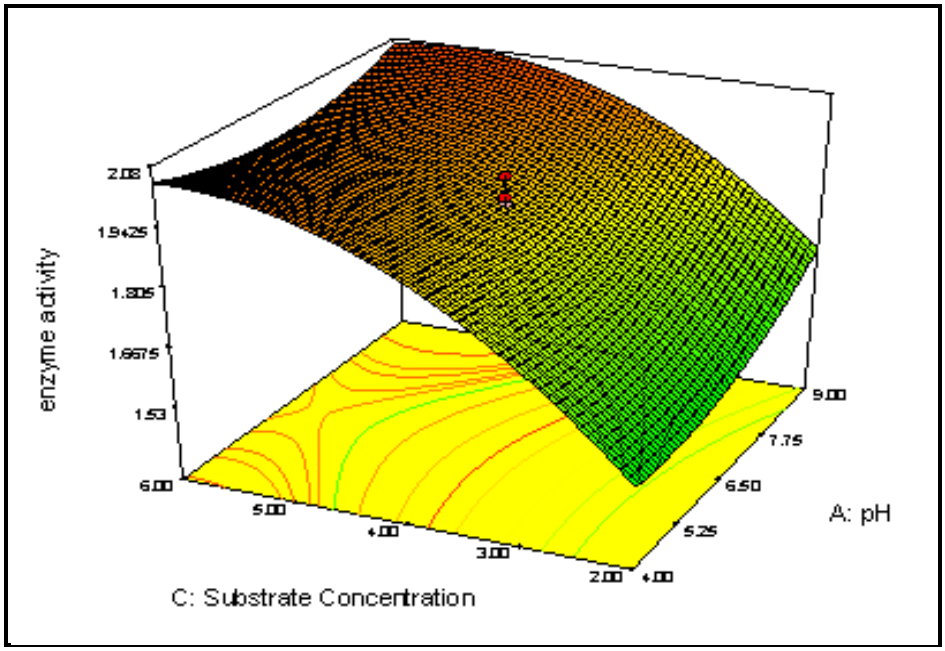

Figure 4.11: Response surface plot from model equation – effect of substrate concentration and temperature.

Figure 4.12 shows the contour of response surface plot for effect of temperature and substrate concentration interaction to amylase activity and Figure 4.13 shows the response surface plot that obtained from the software. Based on Figure 4.13, substrate concentration and temperature affect the amylase activity, and high substrate concentration used resulted in high amylase activity. However, temperature had a slight effect on amylase activity.

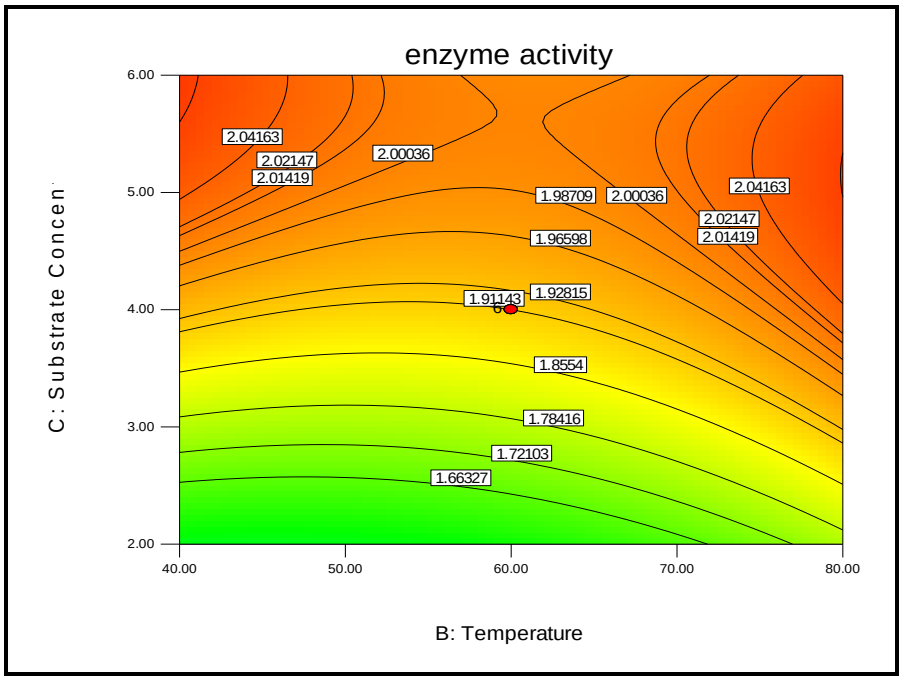

Figure 4.12: Contour plot of amylase activity from model equation – effect of substrate concentration and pH.

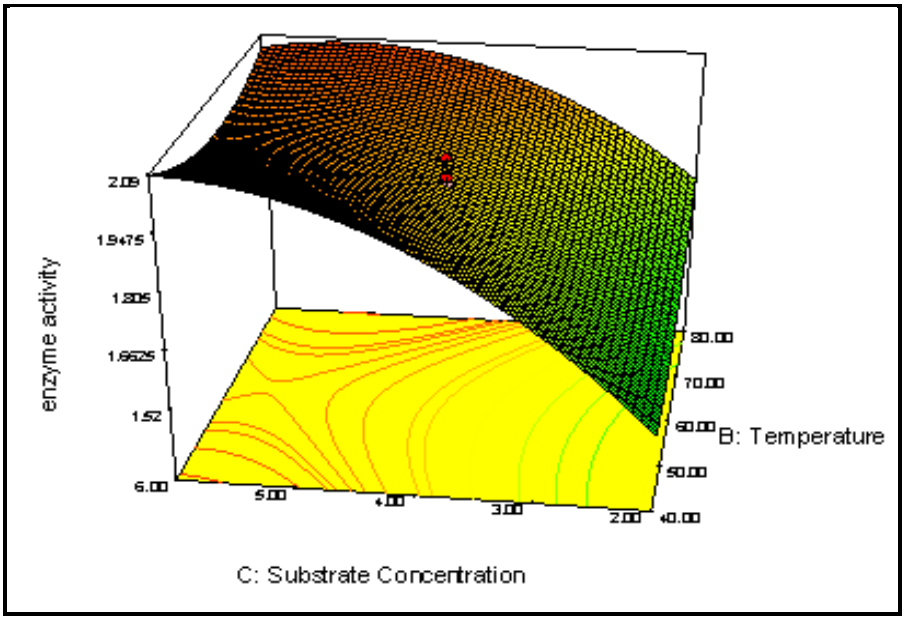

Figure 4.13: Response surface plot from model equation – effect of substrate concentration and temperature.

4.2.1 Optimization of Point Prediction and Confirmation

Based on model obtained from response surface methodology, an optimization point was predicted by software. The suggested optimization points for pH, temperature and substrate concentration were 8.90, 40.76 °C and 5.46% (w/v), respectively. The experimental works was conducted to validate the optimization points. The result obtained was 2.01511 U/ml of enzyme activity compared with predicted value which was 2.1656 U/ml. Differences between predicted value and experimental result was 6.95% which can be accepted.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The research on optimization of enzymatic reaction through design of experiment was successfully carried out. By using 2^4 full factorial designs, the significant parameters that affecting enzymatic reaction of amylase enzyme were able to determine. The significant parameters that affecting enzymatic reaction obtained were temperature, pH and substrate concentration. The objective of this research which was to optimize parameter for enzymatic reaction for enzyme amylase by using design of experiment (DOE) was also achieved. The optimized value for pH, temperature and substrate concentration was 8.90, 40.76 °C and 5.46% (w/v), respectively. The experimental design was proven to be tool for optimization process with less number of experiments and more accurate optimum value.

5.2 Recommendation

To obtain high conversion of starch to glucose, further study on optimization needs to be carried out. Studies on effect of incubation time, agitation rate and effect of metal ion on enzymatic reaction can improve the rate of enzymatic reaction. Optimization of many parameters will give the best optimum condition for enzymatic reaction.

In order to find the value of range of parameter that affecting enzymatic reaction, the conventional method can be used. By using one factor at a time technique, the range of parameter can be predicted. This will make sure the optimum value for particular parameter is still in range and make the optimization process more effective and accurate.

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APPENDIX

Appendix A1

Buffers Preparation for Enzyme Assay

1. Sodium acetate buffer, 0.1 M

Solution A: 5.775 ml glacial acetic acid per liter (0.1 M).
Solution B: 8.2 g of sodium acetate per liter (0.1 M).

Table A-1: Preparation of 0.1 M Sodium Acetate Buffers.

Desired pH	Solution A (ml)	Solution B (ml)
4.0	41.0	9.0
5.0	14.8	35.2

2. Potassium phosphate buffer, 0.1 M

Solution A: 13.6 g of KH_2PO_4 per liter (0.1 M).
Solution B: 22.8 g of K_2HPO_4 per liter (0.1 M).

Referring to Table A-2 for desired pH, mix the indicated volumes of solutions A and B.

Table A-2: Preparation of 0.1 M Potassium Phosphate Buffers.

Desired pH	Solution A (ml)	Solution B (ml)
6.0	87.7	12.3
7.0	39.0	61.0
8.0	5.3	94.7

3. Glycine-NaOH buffer

Solution A: 7.51 g of glycine per liter (0.1 M).
Solution B: 0.1 M NaOH

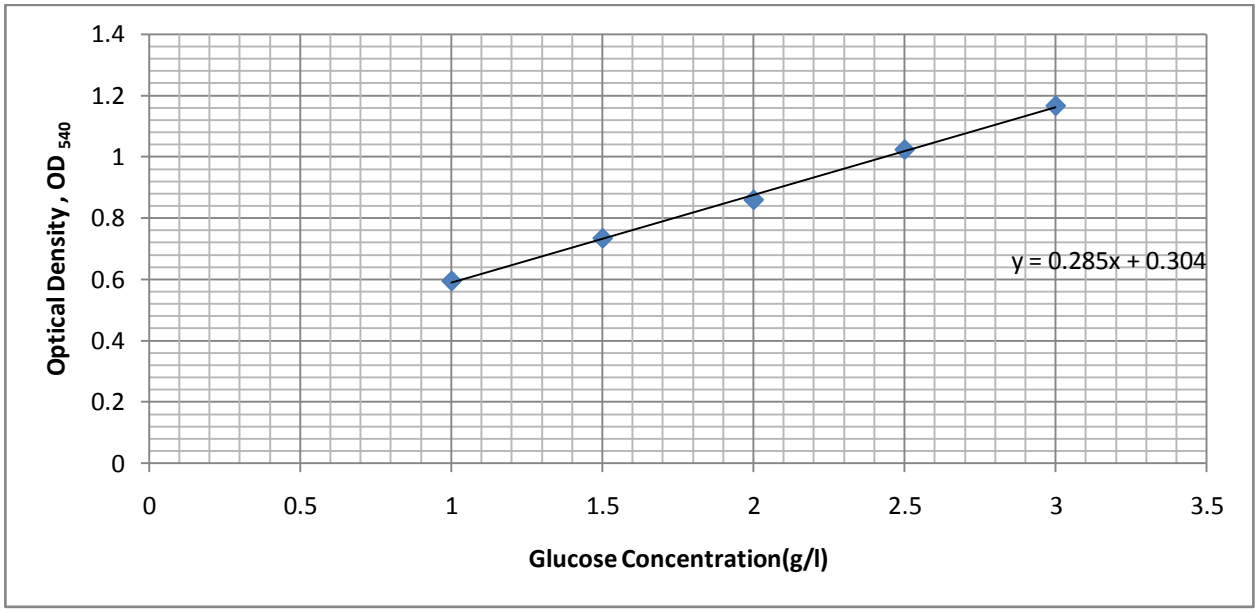
50 ml of Solution A + x ml of Solution B.

Table A-3: Preparation of Glycine-NaOH.

Desired pH	**x (ml)**
9.0	8.8
10.0	32.0

Appendix A2

Standard curve of glucose concentration



Appendix A3

Calculation of amylase activity

Enzyme activity defined as the amount of enzyme that release 1 µmol of reducing sugar per minute. Time for incubation was 15 minutes.

$$Amylase\ Activity\ \left({}^{\mathrm{U}}/_{\mathrm{ml}}\right) = \frac{[G] \times 10^{6}}{15000 \times MW}$$

Where [G] is glucose concentration (g/l)

MW is molecular weight of glucose