OPTIMIZATION OF PULLULANASE ACTIVITY THROUGH DESIGN OF EXPERIMENT (DOE)

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ABSTRACT

Enzyme has been used widely in industries today due to its capability in catalyzing various types of biochemical reactions. However, the high cost of enzyme gives problems to the enzyme industries. A research was done to optimize the pullulanase activity from *Bacillus Acidopullulyticus* using experimental design. Two level Full Factorial design was applied in the screening process of four parameters for pullulanase activity and only three parameters were recognized as significant and effective factors for pullulanase activity. The three significant parameters were pH, enzyme and substrate concentration. Thus the three parameters were selected for further optimization studies using response surface methodology (RSM) in Design Expert version 7.1.6 software with Central Composite Design (CCD) mode. Thirty four sets of experiments with different parameter values were suggested. The predicted optimum values with R^2 of 0.8729 for pH, enzyme concentration, substrate concentration and pullulanase activity were 6.60, 11.11 U/ml, 4.44% (w/v) and 3.17 U/ml respectively. One validation experiment was run using the optimized parameter and resulted 3.05 U/ml pullulanase activities as compared to the suggested values of 3.17 U/ml. As a conclusion, this research was successful to increase the pullulanase activity with a minimum numbers of experiments.

ABSTRAK

Enzim telah digunakan meluas di dalam industri-industri sekarang disebabkan keupayaannya dalam memangkinkan pelbagai jenis reaksi biokimia. Walaubagaimanapun, kos enzim yang sangat tinggi menjadi punca masalah kepada industri enzim. Satu kajian telah dilaksanakan untuk pengoptimuman aktiviti pululanase dari *Bacillus acidopullulyticus* menggunakan kaedah reka bentuk. Reka bentuk penuh faktorial dua peringkat telah digunakan dalam proses saringang empat parameter untuk kajian aktiviti pullulanase dan hanya tiga parameter telah dikenalpasti sebagai faktor-faktor penting dan berkesan dalam aktiviti pululanase. Tiga parameter penting yang memberi kesan kepada aktiviti pullulanase adalah pH, kepekatan enzim dan substrat. Tiga parameter tersebut telah dipilih untuk proses pengoptimuman dengan menggunakan kaedah permukaan sambutan (RSM) menggunakan mod reka bentuk komposit pusat dengan bantuan perisian Design Expert 7.1.4. Tiga puluh empat ekperimen telah disarankan dengan menetapkan nilai-nilai parameter yang berbeza. Model (R^2 = 0.8729) mencadangkan nilai optimum untuk pH, kepekatan enzim dan susbtrat serta aktiviti pullulanase adalah 6.60, 11.11 U / ml, 4.44% (b/i) dan 3.17 U/ml masing-masing. Ekperimen pengesahan telah dilaksanakan dengan menggunakan parameter optimum yang telah dicadangkan dan menghasilkan aktiviti pullulanase sebanyak 3.05 U /ml dengan aktiviti sebanyak 3.17 U/ml yang dicadangkan oleh model. Kesimpulannya, kaedah ini telah berjaya untuk meningkatkan aktiviti pullulanase dengan bilangan eksperimen yang minimum.

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF APPENDICES

LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
CCD	-	Central composite design
OFAT		One-Factor-at-Time
DNS	-	Dinitrosalicylic
DOE		Design of Experiment
g	-	Gram
g/L	-	Gram per liter
L	-	Liter
M	-	Molar
mg	-	Milligram
min	-	Minutes
µM	-	Micromolar
MW	-	Molecular weight
OD_{540}	-	Optical density at 540nm
RSM	-	Response surface Methodology
T	-	Temperature
U	-	Unit for enzyme activity
U/ml		Unit per mililitre
^{o}C	-	Degree Celcius
%	-	Percentage
w/v		Weight per Volume
b/i		berat per isipadu

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In modern technology era, products and processes are becoming more complicated. As the cost of experimentation increase rapidly, it is becoming impossible for the researchers, who is already tighten by resources and time, to examine the numerous factors that affect these complex processes using trial and error methods (Antony, 2003). So a technique is needed to determine the "vital few" factors in the most efficient manner and then direct the process to its best setting to meet the ever-increasing demand for improved quality and increased productivity (Montgomery, 2001). The technique of Design of Experiment (DOE) provides highly efficient method to reach these objectives. DOE is widely used in research and development, where a large proportion of the resources go towards solving optimization problems in product or process. Experimental design techniques become extremely important in such situations to develop new products and processes in a cost-effective and confident manner.

The general practice of determining these optimum parameters by varying one parameter while others remaining constant and observed the factor impact on the process (Nicosia and Sciacca, 2008). The major disadvantage of single variable optimization in the process is that it does not include interactive effects among the variables. Thus, it does not depict the net effects of various parameters on the reaction rate. At the same time, the conventional methods for multifactor experimental designs are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors (Tanyildzi *et al*., 2005). Thus, the application of statistical experiment design was very important to optimize the chemical reaction effectively and precisely in less time consuming. It was in line this research where the main purpose of this research was to explore the potential of design of experiment to identify and optimize parameters for enzymatic reaction for enzyme pullulanase.

Several studies were reported improvements in the enzymatic reactions of pullulanase where the parameters have been an object of study for pullulanase activity. Subhash (2006) reported that, the pullulanase was obtained higher activity at optimum temperature at 75^{o}C. There are also having other several factors that affect enzymatic reaction of pullulanase including substrates, pullulanase activity and also reaction conditions (temperature and pH). To improve yield and rate of the enzymatic reaction, research has focused on the optimization of hydrolysis process and enhancement of pullulanase activity.

1.2 Problem Statement

During the last few decades, many industrial engineers and researchers perform one-factor-at-a-time (OFAT) experiments to inspect the situations of process improvement and for problem-solving activities. However, OFAT experiments can be proved as an ineffective and unreliable and leading to false optimal condition. Besides that, they often consists a lot of "trial and error", intuition, presumption and experience for their success (Clements, 1995). This type of experimentation needs large resources to gain a limited amount of information about the process.

While design of experiments (DOE) is an alternative, more structured approach. Moreover DOE allows engineers and researchers to differ several factors simultaneously in such a way that applicable, reliable, effectively and economically from the experiment (Antony *et al.*, 2003).

Despite being impossible to do the OFAT experiments in this research of optimization pullulanase activity, the design of experiments is used as it gives more advantages than OFAT and more precise. Thus DOE allows investigating the importance of interactions among the factors considered for the experiment. DOE also provides a better optimization strategy than the OFAT approach.

1.3 Research objective

The objective of this research is to optimize the enzymatic reaction parameters for pullulanase enzyme by using statistical approach.

1.4 Scope of study

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

1. to identify significant factors that affect enzymatic reaction through full factorial design and
2. to optimize the significant factors via response surface method.

CHAPTER 2

LITERATURE REVIEW

2.1 History

Enzyme is one of the most protein molecules involved in reactions of living organisms which role as catalyst. Without enzyme these reactions would not occur fast enough to sustain life (McKee, 2003). The term of enzyme was introduced by Kiihne in 1878, although the first study of enzyme activity in a test tube was reported by Payen and Persoz in 1833. While during 1890s, Fisher recommended the 'lock and key' model of enzyme reaction, at the same time a mathematical model of enzyme action was proposed by Michaelis and Menten in 1913. In 1947, John H. Northrop, Wendell M. Stanley and James B. Sumner have discovered complex procedure for isolate pepsin. The technique developed by Northdrop and Stanley has been used to crystallize several enzymes (Holumn, 1968).

2.2 Enzyme

All known enzymes are considered to be proteins that catalyze, or speed up a chemical reaction. Enzymes are essential to sustain life because most chemical reactions in biological cells would occur too slowly or lead to different products without enzymes. Like all catalysts, enzymes lower the activation energy for the reaction they are catalyzing as illustrated in Figure 2.1.

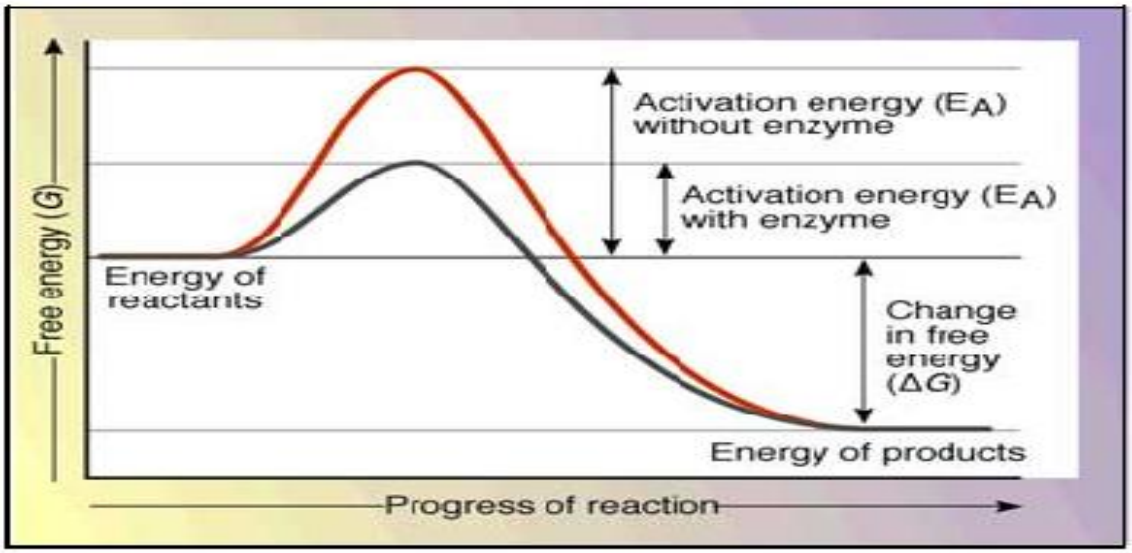

Figure 2.1: Activation energies of reactions with and without enzyme (Bennett and Frieden, 1969)

Enzymes speed up the reactions by a factor of many millions. Thus allowing the reaction to proceed much faster (Holum, 1968). They also remain unchanged by the completion of the reaction and can continue to function. Since enzymes do not affect the relative energy between the products and reagents, they do not affect the equilibrium of the reaction (Smith *et al*., 1997).

Typically, most enzyme names end in “ase” except for some of the originally studied enzymes such as pepsin, rennin, and trypsin. The International Union of Biochemistry (I.U.B) initiated standards of Enzyme Commission or EC numbers which classified enzyme based on the reactions they catalyze.

The top level classification is (McKee *et al*., 2003):

i. EC1 *Oxidoreductases:* oxidation-reduction reactions.
ii. EC2 *Transferases:* transfer a functional group. (e.g. a methyl or phosphate group)
iii. EC3 *Hydrolases:* catalyze the hydrolysis of various bonds.
iv. EC4 *Lyases:* cleave various bonds by means other than hydrolysis and oxidation.
v. EC5 *Isomerase:* catalyze isomerization changes within a single molecule.
vi. EC6 *Ligases:* catalyze bond formation between two substrate molecules.

In this research, pullulanase (pullulan 6-glucanohydrolse, EC 3.2.1.41), a debranching enzyme, was gained importance in starch-processing industry normally for producing smaller product for example high fructose syrup and maltose (Rozaimi *et al.,* 2008). This enzyme became of interest due to its application that could be used together with exo-acting enzymes such as glucoamylase or β-amylase, which are not able to hydrolyze the α-1, 6 glycosidic bonds in polysaccharides and to improve the yield of the final starch hydrolysis product (Reddy *et al.,* 2000).

2.3 Properties of Enzyme

Enzymes perform their work at moderate temperature and are highly specific in the reactions that each one catalyzes (McKee, 2003). For example, enzyme pullulanase cleave the branches of amylopectin molecules and produce thereby chains of amylose. Thus, the enzyme can breakdown the only amylase enzyme. This characterization shows the enzyme only can present one specific work with very few side effects (McKee, 2003). Enzymes also like all catalysts cannot alter the equilibrium of the reaction, but they can increase the rate towards equilibrium.

The other major property of enzyme activity and stability is its sensitivity to temperature. Seeing as enzymes are biochemical catalysts, they are sensitive in varying degrees of temperature. Raising temperatures of the environment commonly multiplies the degree of activity by the enzyme. Generally the ideal optimal temperatures for enzymatic reaction are in the range of 40 to 60°C (Aehle, 2004). Once an optimum temperature was reached, rapid degradation of the enzyme with concurrent and irreversible loss in activity had occurred. Enzyme also works well under its optimum pH. While the value of pH is less or more than optimum value, the enzyme activities will also decrease. Typically for the most enzymes, the pH optimums are between ranges from 5 to 7 (Aehle, 2004).

2.4 Factors affecting enzymatic reaction

Any environmental or chemical factor that disturbs protein structure may change enzymatic activity. Despite of these, several factors affect the rate at which enzymatic reactions proceed such as temperature, pH, enzyme concentration and substrate concentration. The optimization process is conducted effectively to make better understanding on how these factors affect enzymatic reaction.

2.4.1 Temperature Effects

The temperature dependence on enzymatic reaction exhibits an optimum because the thermodynamic increase of reaction rate. Figure 2.2 shows that when the temperature rises, molecular motion hence collisions among enzyme and substrate will increase. The optimum temperature is generally between 40℃. Otherwise, there are enzymes with other different optimum temperature, such as 80°C. But as enzymes are proteins, there is an upper limit beyond which the enzyme becomes denatured and ineffective.

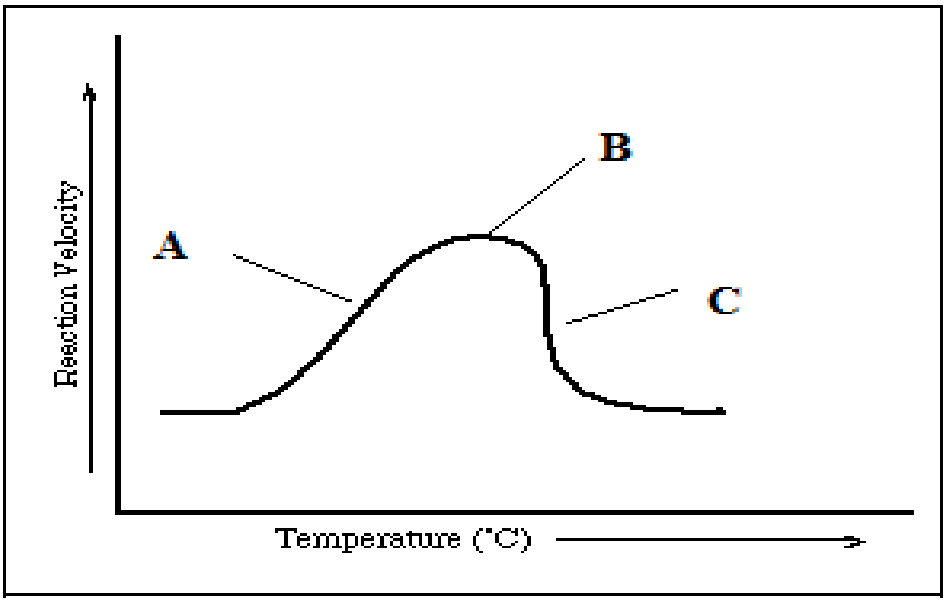

Figure 2.2: Effect of temperature on enzyme activity. A: Increasing activity, B: The optimum temperature for enzyme activity, C: Lost of enzyme activity that known as denaturation (Bennett and Frieden, 1969).

2.4.2 Effects of pH

The conformation of a protein is influenced by pH and as enzyme activity is crucially dependent on its conformation, its activity is likewise affected. An enzyme's conformation can be affected when the ionization state of its amino acid side chains is altered by pH changes. Affects of ionic and hydrogen bonds will cause responsible for holding a protein in its biologically active conformation. The optimum pH is the point where the enzyme is most active. This is illustrated in Figure 2.3.

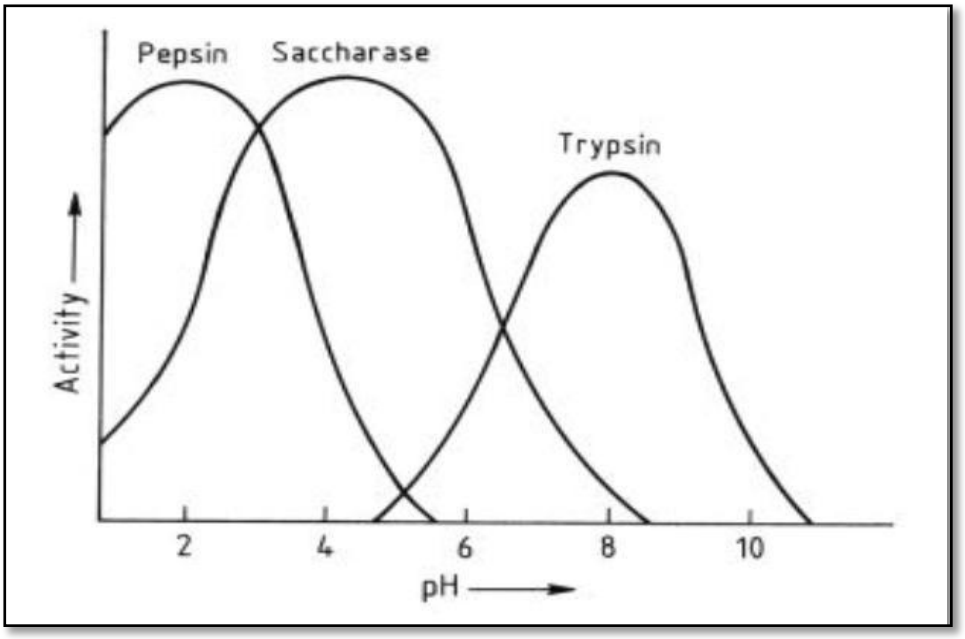

Figure 2.3: Effect of pH on enzyme activity. Different enzymes exhibit different optimum pH due to ionic strength and type of buffer (Aehle, 2004).

2.4.3 Effects of Enzyme Concentration

The amount of enzyme present in a reaction is measured by the activity it catalyzes. Rate of reaction is directly proportional to the enzyme concentration. The percentages for formation of enzyme-substrate-complex will increase when the concentration was increased therefore the probabilities for reaction to occur will be higher. While the substrate amount should be present in excess amount to make sure the enzymatic reaction is independently to substrate. In Figure 2.4, the product concentration is proportional with time and the reaction is to be zero order. The enzymatic reaction was affected when the amount of enzyme was increased. Thus the amount of enzyme increase, the rate of enzymatic reaction increase with the increase of enzyme-substrate-complex formed (Worthington, 1972).

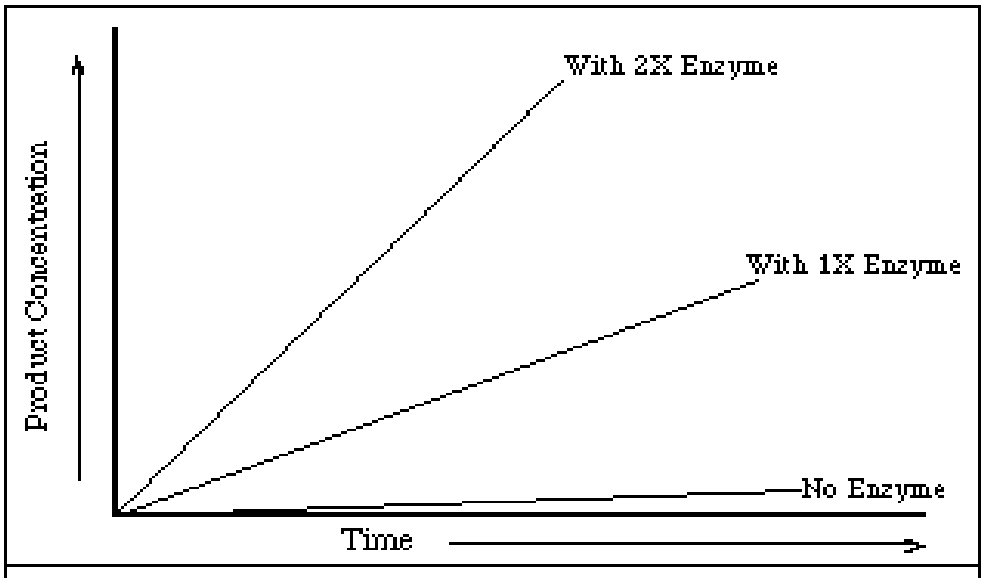

Figure 2.4: Effect of amount of enzyme to product concentration. As the amount of enzyme increase rate of reaction also increase (Worthington, 1972).

2.4.4 Effects of Substrate Concentration

When the enzymes are available in excess, the rate of reaction is proportional to the amount of substrate. But as the maximum rate of enzymatic reaction had been reached, it is show that the entire available enzyme has been converted to enzyme-substrate (ES), which is the enzyme substrate complex (Worthington, 1972). . This maximum is set by the amount of active enzymes. If the substrate amount is further increased the enzyme concentration will become the limiting factor for the reaction.

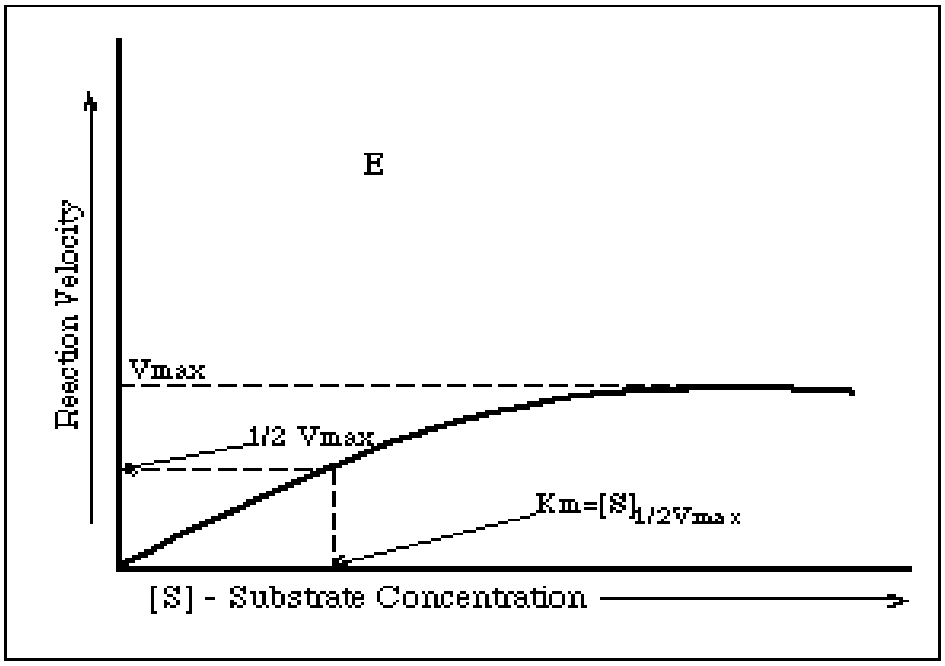

Figure 2.5: Effect of substrate concentration on enzyme activity (Holum, 1968)

2.5 Design of Experiments (DOE)

Design of experiment (DOE) has grown out of the need to plan efficient experiments in agriculture in England during the early part of the 20th century. The first statistician to consider a methodology for the design of experiment (DOE), is Sir Ronald A. Fisher from the Rothamsted Agricultural Field Research Station in London, England (Montgomery, 2001). He used DOE which could differentiate the effect of fertilizer and the effect of other factors. It allowed him to illustrate the most important means of experiment design:

i. Randomization – The process of making something random
ii. Replication – Repeating the creation of the phenomenon, so that the variability associated with the phenomenon can be estimated.
iii. Blocking – The arranging of experimental units in groups (blocks) which are similar to one another.
iv. Orthogonality – Means perpendicular, at right angles or statistically normal.
v. Use of factorial experiments instead of the one-factor-at-a-time method.

DOE has been widely accepted and applied in biological and agricultural fields.DOE also helps to optimize a product or process. This is achieved by determining which factors in a process may have the greatest effect on the response. In otherwise, it provides a powerful mean to achieve breakthrough improvement in product quality and process efficiency (Antony, 2003).

2.5.1 Type of DOE

Generally there are three big groups of design method in Design of experiment since four eras have been recognized in the modern development of statistical experimental design such as Two Level Full Factorial design, Taguchi and Response Surface Methodology (RSM).:

2.5.1.1 Two Level Full Factorial design

The factorial design (full or fractional factorial design) has widely accepted that most commonly used in experimental designs in manufacturing companies. It would enable an experimenter to explore the set of input variables or factors (such as processing of design parameters) on responses (such as yield). A proper designed experiment can minimize the number of experimental runs and used of random sampling.

Many experiments involve the study of many effects/factors or searching the optimum condition that used a lots of parameters combination. In general, factorial designs are most efficient for this type of experiment (Antony, 2003). By a factorial design, each complete trial or replication of the experiment all possible combination of the levels of the factors can be investigated

The advantage of factorial design can be easily illustrated. They are more efficient than one-factor-at-a-time (OFAT). Furthermore a factorial design is necessary when interaction may be present to avoid misleading conclusion.