OPTIMIZATION OF MEDIUM FORMULATION ON PRODUCTION OF RECOMBINANT CHITINASE IN *Escherichia coli*

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OPTIMIZATION OF MEDIUM FORMULATION ON PRODUCTION OF RECOMBINANT CHITINASE IN *Escherichia coli*.

MOHD FARIS BIN MOHD YUNUS

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

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30 April 2010

I declare that this thesis entitled "Optimization of Medium Formulation on Production of Recombinant Chitinase in *Escherichia coli*." is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Special Dedication to mak, abah and family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and belief in me.

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ABSTRACT

Chitinase is enzyme that hydrolyzing chitin to produce N-acetyl-glucosamine. Chitinase can be found in bacteria, fungi, higher plant, insect and some vertebrates. There are a lots of applications of chitinase as the demands of the enzyme is rising high in the market due to usage in pharmaceutical, biopesticides or food industry. For this study, chitinase enzyme was expressed in recombinant bacteria using *Escherichia coli* as a host. The effect of various medium on expression of chitinase in *Escherichia coli* was conducted in this research. Five medium were studied. LB, TB, SB, SOB and 2x YT were screened. LB medium gave the best result of highest chitinase enzyme activity. Each component of LB medium was study to determine the optimize amount of composition to produce the highest enzyme activity. The best range of every composition obtained from the conventional method was 3.0 g/l to 7.0 g/l for sodium chloride, 1.5 g/l to 4.5 g/l for yeast extract and 10 g/l to 14 g/l for tryptone. The optimization was done using response surface methodology (RSM) conducted by software named Design Expert. The optimal medium composition for high soluble recombinant chitinase was determined as 3.63 g/l of sodium chloride, 4.50 g/l of yeast extract and 13.11 g/l of tryptone. From the experimental, the enzyme activity after optimization was achieved 2.291 U/ml compared to predicted response of 2.409 U/ml. This result shows increment of the activity for 82% than before the optimization which is 0.411 U/ml. It shows that the optimization of the medium formulation to improve the expression and production of chitinase was successfully conducted by using RSM.

ABSTRAK

Enzim kitinase menghidrolisis kitin untuk menghasilkan *N-acetyl-glucosamine*. Kitinase boleh ditemui dalam bakteria, kulat, tumbuhan tinggi, serangga dan beberapa haiwan vertebrata. Terdapat banyak aplikasi kitinase sebagai enzim yang mempunyai permintaan tinggi di pasaran kerana penggunaannya di farmasi, biopesticides dan industri makanan. Untuk kajian ini, enzim kitinase diekspreskan dalam rekombinan bakteria *Escherichia coli* yang dijadikan sebagai perumah. Tujuan penyelidikan adalah untuk mengoptimumkan penghasilan rekombinan kitinase. Pengaruh pelbagai media terhadap ekspresi enzim kitinase pada *Escherichia coli* diselidiki. Lima medium telah dikaji iaitu LB, TB, SB, SOB dan 2x YT. LB medium memberikan hasil yang terbaik dengan menghasilkan aktiviti enzim kitinase tertinggi. Setiap komponen medium LB dikaji untuk mendapatkan nilai komposisi yang optimum untuk menghasilkan aktiviti enzim tertinggi. Julat terbaik untuk setiap komposisi yang diperolehi daripada kaedah biasa adalah 3.0 g/l hingga 7.0 g/l untuk natrium klorida, 1.5 g/l hingga 4.5 g/l untuk ekstrak ragi dan 10 g/l hingga 14 g/l untuk trypton. Proses pengoptimuman dilakukan dengan menggunakan kaedah gerak balas permukaan (RSM) yang dijalankan menggunakan perisian Design Expert. Komposisi media yang optimal untuk penghasilan kitinase rekombinan ditentukan sebagai 3.63 g/l natrium klorida, 4.50 g/l ekstrak ragi dan 13.11 g/l trypton. Daripada uji kaji, aktiviti enzim setelah optimasi medium mencapai 2.291 U/ml berbanding nilai anggaran oleh perisian iaitu 2.409 U/ml. Keputusan ini menunjukkan peningkatan aktiviti sebanyak 82% daripada sebelum pengoptimuman yang hanya menghasilkan 0.150 U/ml. Ini menunjukkan bahawa pengoptimuman medium untuk meningkatkan ekspresi dan penghasilan kitinase telah berjaya dijalankan dengan menggunakan RSM.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of Variance
CCD	-	Central composite design
DNS	-	Di-Nitro Salicylic Acid
g	-	Gram
g/L	-	Gram per litre
hr	-	Hour
IPTG	-	Isopropyl-β-D-thiogalactoside
mg	-	milligram
mg/L	-	milligram per liter
Min	-	minutes
mL	-	mililiter
mM	-	milimolar
OD	-	Optical density
PMSF	-	Phenylmethylsufonylfluoride
R	-	Correlation coefficient
R^2	-	Regression coefficient
RSM	-	Response surface methodology
rpm	-	Round per minute
SSF	-	Solid substrate fermentation
U/mL	-	Unit enzyme activily per mililiter
%	-	percentage
°C	-	degree Celsius
µL	-	microliter
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Chitin, a β -1, 4-linked homopolymer of N-acetylglucosamine (GlcNAc), is the main structural material in the fungal cell walls, insect exoskeletons and crustacean shells. The β -1, 4- glycoside bond in chitin is hydrolyzed by chitinases, that exist in many species, including bacteria, fungi, insects, even plants and human beings in which chitin are not found (Fan *et al*., 2007).

Chitinase is the enzyme that degrades chitin. Chitin is a linear polymer of N-acetyl glucosamine residues linked by β -1, 4 glycosidic bonds and is a component of fungal cell walls or arthropod integuments. Endochitinase and N-acetylhexosaminidase are two major enzymes of the chitinase system. Chitinolytic enzymes have broad range of applications. Use as biocontrol agents and conversion of waste chitin produced by shellfish processing industry to single cell protein. It also use in cytochemical localization of chitin/chitosan using chitinase–chitosanase–gold complex, fungal protoplast technology and preparation of chitooligosaccharides (Vaidya *et al*., 2003).

Bacterial chitinases are considered to be important in digestion and utilization of chitin as a carbon source and they are key enzymes for the recycling of chitin in the environment. Thus far, a number of reports have been published on the molecular cloning and nucleotide sequencing of chitinase genes from aerobic bacteria such as *Bacillus circulans, Aeromonas* sp., *Alteromonas* sp. and an anaerobic bacterium *Clostridium paraputrificum* (Morimoto *et al*., 2001).

The emergence of a new biotechnological industry utilizing recombinant DNA technologies for the production of highly specific biomolecules has increased the demand for sophisticated tools such as restriction enzymes, which enable the researchers to cut and paste DNA fragments (Nikerel *et al*., 2005).

Since the early development of recombinant DNA technology, *E. coli* has been widely used as a host for high-level expression of recombinant proteins. Large-scale productions of valuable proteins in this expression system are usually achieved using a two-stage process. In the first stage, cells are grown to a high cell density under favorable growth conditions in which protein synthesis is kept at minimum via tightly regulated promoter systems. This is followed by a second stage in which high-level expression of the recombinant protein is achieved upon induction (Nikerel *et al*., 2006).

E. coli grows in both rich complex organic media as well as in salt-based chemically defined media as long as an organic carbon source is provided. Through the type and concentration of ingredients used, cultivation medium composition directly dictates the amount of biomass produced and therefore can dramatically influence the performance of microbial processes (Nikerel *et al*., 2005).

In recent years, the cloning and expression of the chitinase gene and the construction of recombinant strains with new capacities have been one of the interesting areas of chitinase studies and applications Chitinase genes have been cloned and characterized from many microorganisms including *Serratia marcescens, Alteromona, Enterobacter agglomeron, Streptomyces olivaceoviridis, Streptomyces iividans, Aeromonas caviae*, and *Bacillus circulans,* some of which were either transformed into plants and bacterial strains to increase their ability to control fungal phytopathogens or were highlevel expressed in *Escherichia coli* cells to enhance the activity of *Bacillus thuringiensis* to control pests (Yong *et al*., 2006).

Other microorganisms, including bacteria such as *Bacillus lichiniformis, Bacillus pabuli, Nocardia orientails, Vibrio alginolyticus,* and many species of fungi such as: *Myrothecium verucaria, Stachybotrys elengans, Streptomyces cinereoruber, Streptomyces lydicuis, Trichoderma harzianum, Trichoderma viride, Verticillium lecanii* have a chitinase-producing ability (Liu et al., 2003). Fan *et al*. (2007) also reported that the enzyme also can be express in *Escherichia coli* and *Pichia pastoris.*

Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions have successfully been used in the optimization of bio processes (Nikerel *et al*., 2005). The concept of RSM has eased process development and has been of significant use at industrial level (Nawani and Kapadnis, 2005).

This RSM method can be used in different ways of optimization process. The method is widely used for optimizing the medium components. Akhir *et al*., (2009) had successfully optimized medium for chitinase enzyme production from shrimp waste. Vaidya *et al*., (2003) also had done optimized medium components for the production of chitinase by *Alcaligenes xylosoxydans* culture. Also, the method can be used to optimize culture condition. Pan *et al*., (2008) has effectively optimized the culture condition enhance *cis*-epoxysuccinate hydrolase production in *Escherichia coli.*

1.2 Problem Statement

Interest in chitinase enzyme application has markedly increased in agricultural industries, but the price is also high due to the complicated process. The switch to chitinase-producing recombinant *E. coli* is seen here as an economic alternative towards higher productivity and easier downstream purification. Growing *E. coli* to high density is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method. Exploring the growth limits of microorganisms in general and *E. coli* in particular, engaged industrial microbiologists many years before it was possible to convert *E. coli* to a "production machine" for heterologous proteins. Thus, optimization of medium for *E. coli* is essential to obtain the highest density of cell growth. Today, techniques to obtain the highest possible density of productive *E. coli* in submerged cultures are well developed.

1.3 Objectives

The main objective in this study is to optimize the production of recombinant chitinase in *E. coli* using Response Surface Methodology (RSM).

1.4 Scopes of Research

The scopes of the study are:

a) To study the effect of various medium on expression of chitinase in *E. coli*
b) To study the effect of medium formulation using the best medium
c) To optimize the medium formulation on production of chitinase in *E. coli* using RSM

1.5 Rationale and Significance

This research will provide the alternative production of chitinase. It helps to increase the production of chitinase while reducing the production cost of chitinase by using the low cost material. The usage of *E. coli* as a raw material in producing chitinase is actually helping reducing the cost as to o. The efficiency of the production is depending on the growth of the *E. coli*. Growing the *E. coli* that contains the recombinant chitinase to high cell density before harvest it will increase the production of chitinase. This research will help to find the optimum medium formulation for the growth of *E. coli* to high cell density.

CHAPTER 2

LITERATURE REVIEW

2.1 Chitinase

Chitinase hydrolyzes the β -1,4-linkage of chitin and is known to be produced by a wide range of organisms, found in bacteria, fungi, higher plant, insect and some vertebrates Chitin is an insoluble β -1,4-linked polymer of N-acetylglucosamine (GlcNAc) and is one of the most abundant biomasses on earth, second only to cellulosic materials (Morimoto *et al*., 2001).

Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, l-glutamine, l-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al*., 2006).

The biological functions of chitinases in various organisms are diverse. Plant chitinases were involved in plant defense mechanism against fungal pathogens, however fungal chitinases were associated with autolytic morphogenetic and pathogenesis roles (Fan *et al*., 2007).

2.1.1 Type of Chitinase

Chitinases can be divided into two categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain and endochitinases, which hydrolyse internal β -1,4-glycoside bonds. Many plant endochitinases especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity. Chitinases have been reported from several species of plants as two isoforms, acidic and basic. The acidic and basic isoforms of chitinase are induced in plants, in response to pathogen attack and other environmental stimuli and are also expressed in certain tissues of plants during normal development. There are several reports that chitinases isolated from monocot and dicot plants have been shown to inhibit the growth of fungi in vitro (Kirubakaran and Sakthivel, 2007).

The mechanism of hydrolysis of family 18 chitinases has been studied in some detail and these chitinases hydrolyze chitin via a substrateassisted mechanism, through an oxazoline ion intermediate, involving the carbonyl group of the aceta amido group of chitin. Therefore, a single acidic residue is sufficient for catalysis (Kuttiyawong *et al*., 2008).

A previous study revealed that *Serratia marcescense* produces three distinct chitinase enzymes, ChiA, ChiB and ChiC that favor different modes of catalysis in terms of exo- or endo-type. ChiA and ChiB are processive chitinases that degrade chitin chains in opposite directions, while ChiC is a non-processive endo chitinase. ChiA, a family 18 chitinase, from S. marcescense has been extensively studied and exposed aromatic residues on the surface of ChiA has been identified and suggested to be involved in binding and guiding of the substrate into the catalytic cleft during the processive mode of catalysis. Changing these Trp residues to Ala residues reduces the ability of ChiA to bind to its substrate, as well as the activity of the enzyme on insoluble substrates. A proposed model of hydrolysis of chitin by ChiA suggests it is an exo-chitinase hydrolyzing chitin strand from the reducing end (Kuttiyawong *et al*., 2008).

2.1.2 Classification of Chitinase

Plant chitinases are a diverse group of enzymes with respect to their structure, cellular localization and enzymatic properties. In dicots, the chitinase gene family has been broadly classified into three groups. Class I chitinases are basic and contain a cysteine rich N-terminal domain with putative chitin binding properties. They are usually localized in the vacuole and are potent growth inhibitors in vitro of many fungi. Class II chitinases are generally acidic and extracellular and can be detected in the apoplastic fluid or culture medium of protoplasts. They are not thought to be antifungal either alone or in combination with other proteins. Class III chitinases are extracellular hydrolases whose conserved catalyticdomain amino acid sequence difers from the conserved sequence of class I or II chitinases (Kirubakaran and Sakthivel, 2007).

Chitinase belong to the O-glycosyl hydrolases (GH), a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. A new classification of glycosyl hydrolases based on similarities in amino acid sequences has been put forward. This groups the five known chitinase classes into two GH families, each of which exhibits a strict conservation of the catalytic machinery and enzymatic mechanism. Chitinases of family 18 (formerly class III and V) have been well studied, with information available on the three-dimensional structure and the biochemistry of the enzyme reaction. They operate with retention of the anomeric configuration, whereas family 19 members (class I, II, and IV) invert the configuration at the hydrolysis site. Family 18 of GH (GH18) comprises chitinases from various species, including bacteria, fungi, nematodes, arthropods, mammalians and plants, but also several mammalian, insect and plant proteins lacking chitinolytic activity due to substitution of a critical acidic amino acid in the catalytic centre. This new class of proteins has been called chitinase-like proteins (CLPs). Recently, we identified the first lophotrochozoan CLP from the oyster *Crassostrea gigas* (Badariotti *et al*., 2007).

From previous studies due to amino acid sequence similarity, families 18, 19 and 20 of glycosyl hydrolases can refer to group of chitinolytic enzyme. Chitinase contain from bacteria, fungi, viruses, animals and some plant chitinases are referring to evolutionary term of family 18. While according to family 19, there are consist of plant chitinases (class I, II and IV) (Hart *et al.,* 1995). Both of two families are not share amino acid sequence similarity. However, family 20 is includes the β-*N*-acetylhexosaminidases from human, bacteria and *Streptomycetes* (Dahiya *et al.,* 2006).

2.1.3 Function of Chitinase

In order production of chitinase from organism, there are several function that can be used by organism itself and also industrial sectors. In bacteria, chitinase play roles in nutrition and parasitism while involving morphogenesis in fungi, protozoa and invertebrates. In addition, chitinase are also involved in the defence mechanism of plant and vertebrates (Reetarani *et al.,* 1999).

Furthermore, chitinases are enzyme that capable to hydrolyze chitin to its monomer N-acetyl glucosamine (GlcNAc) (Felse and Panda, 1999). In bacteria chitinase are considered primarily to digest and hydrolyse chitin into a carbon and nitrogen nutrient. On the other hand, chitinase act like catalyst in chitin degradation (Cohen and Chet, 1998).

Fungal chitinase are important for surviving of producing organism itself. This is because they are involved in important morphogenic processes such as spore germination, hyphal elongation and hyphal branching (Kuranda and Robbins, 1991; Takaya *et al.*, 1998).

2.1.4 Chitinase Production

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method (Nampoothiri *et al*., 2004).

Production of chitinase is widespread in variety of organisms such as bacteria, fungi, actinomycetes, yeast, plants, protozoans, coelenterates, nematodes, molluscs, arthropods and humans. Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, l-glutamine, l-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al*., 2006).

Solid substrate fermentation (SSF) has recently gained importance for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation. Among the various groups of microorganisms used in SSF, filamentous fungi are most widely exploited because of their ability to grow on complex solid substrates and production of wide range of extracellular enzymes. SSF has been reported to be an economical alternative to submerged fermentation for the production of high titers of proteases and amylases by *Streptomyces rimosus*. Very few reports are available on production of chitinases under SSF conditions, while voluminous literature exists on the production of chitinases by fungi in submerged fermentation (Patidar *et al*., 2005: Nampoothiri *et al*., 2004).

In recent years, there has been a surge in the researches regarding chitinase producing *B. thuringiensis* strains: First, the selection of chitinase-producing *B. thuringiensis*. Second, cloning and expression of chitinase-encoding genes of other microbial chitinase in *B. thuringiensis* to enhance the entomotoxicity value. Third, production of endogenous chitinase from *B. thuringiensis* as well as cloning, sequencing of chitinase genes or characteristics of chitinases from some *B. thuringiensis* strains such as *B. thuringiensis* var. kurstaki, *B. thuringiensis* var. parkitani, *B. thuringiensis* var. kenyae, *B. thuringiensis* var. israelensis, *B. thuringiensis* var. alesti and *B. thuringiensis* var. sotto (DangVu *et al*., 2009).

Morimoto *et al*., (2001) stated that *C. paraputrificum* is a strictly anaerobic, sporogenic, Gram-positive bacterium. It produces several chitinases when grown with ball-milled chitin as the sole carbon source. Recently, Morimoto *et al*., (2001) have cloned and sequenced the *chiA* and *chiB* genes encoding the major chitinases in the culture broth of *C. paraputrificum* M21. These chitinase genes form an operon and the domain architectures of ChiA and ChiB are identical, *i.e.*, they comprise a family 18 catalytic domain of glycoside hydrolases, two cadherin- like domains, and a family 12 carbohydrate-binding module in this order.

2.2 *Escherichia Coli* Expression System

Early studies on high cell density growth of aerobic gram-negative bacteria, including *E. coli* were performed either to investigate the limits of bacterial growth in liquid cultures or to obtain large quantities of exponentially grown *E. coli* needed for biochemical studies. During the 1980s, when much information on the genetics and physiology of the bacterium accumulated and *E. coli* became the obvious organism of choice for recombinant protein production, much more emphasis was put on its high-density growth. Since then, numerous methods to obtain high-density cultures have been developed, each aiming at providing means to bypass the physiological constrains that prevent bacteria from growing to the limit of physical barriers between solid state and liquid suspension of cells (Shiloach and Fass, 2005).

Growing *E. coli* to high density is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method. Exploring the growth limits of microorganisms in general and *E. coli* in particular, engaged industrial microbiologists many years before it was possible to convert *E. coli* to a production machine for heterologous proteins (Tokuyasu *et al*., 1999).

The recombinant expression system in Escherichia coli is the most efficient and widely used system for recombinant proteins. With the advent of the post genomic era, the necessity for useful expression systems in *E. coli* has been increasing in order to analyze the functional and three dimensional aspects of proteins encoded by an increasing number of genes. For this purpose, various effective approaches, including different expression vectors and affinity tags such as *Schistosoma japonicum* glutathione S-transferase (GST), His-tag, FLAG-tag, *E. coli* maltosebinding protein (MBP)-tag have been developed (Seto *et al*., 2008).

Candida antarctica lipase B has previously been expressed in an active form in the *Escherichia coli* cytoplasm and in the periplasm. Eukaryotic expression has been reported in *Pichia pastoris*, *Saccharomyces cerevisiae*, *Aspergillus oryzae* and in *Nicotiana benthamiana* using a viral vector. Prokaryotic expression of active *Candida antarctica* lipase B reported so far has been remarkably lower in terms of yields in comparison to the eukaryotic expression systems. Genetic modifications of *Candida antarctica* lipase B in eukaryotic systems are time consuming in comparison to prokaryotic systems, as are the protein expression and screening of improved *Candida antarctica* lipase B variants in eukaryotic systems. It is therefore of general interest to obtain a wider understanding of the low yield of *Candida antarctica* lipase B in *E. coli* (Larsen *et al*., 2008).

Studier and colleagues first described the pET expression system, which has been developed for a variety of expression applications. More than 40 different pET plasmids are commercially available. The system includes hybrid promoters, multiple cloning sites for the incorporation of different fusion partners and protease cleavage sites, along with a high number of genetic backgrounds modified for various expression purposes (Sørensen and Mortensen, 2005).

Overexpression of recombinant enzymes in *E. coli* allows the engineering of the enzymes for the study of structure-function relationships by site-directed mutagenesis as well as improvement of the enzymes properties using directed evolution technology. The main limitation of using *E. coli* for secretion of recombinant proteins is that not every protein can be secreted efficiently. Moreover, *E. coli* expression systems are not common for large-scale production of products that are used in food industry because of its potential pathogenicity, only *E. coli* K-12 has been approved for generally recognized as safe (GRAS) status by USA-FDA Food and Drugs Administration (Yamabhai *et al*., 2008).

2.3 Factor Affecting the Production of Chitinase Enzyme

2.3.1 Effect of Culture Medium on Production of Chitinase Enzyme

The growth of *E. coli can* be limited by other nutritional requirements including carbon, nitrogen, phosphorus, sulfur, magnesium potassium iron, manganese, zinc, copper and some growth factors .When growing *E. coli* to low density, all the required nutrients can be added initially into the basal broth. The popular complex Luria Bertani (LB) broth allows the growth of E. coli in a temperature, pH, and oxygen-controlled environment up to a cell density of 1 g/l dcw. To accommodate the nutritional requirements of denser cultures, concentrations of media components must be increased and phosphorus, sulfur and trace elements must be added (Shiloach and Fass, 2005).

Unfortunately, most media ingredients required for growth become inhibitory to *E. coli* when added at high concentrations. It is well established that nutrients such as glucose at a concentration of 50 g/l, ammonium at 3 g/l, iron at 1.15 g/l, magnesium at 8.7 g/l, phosphorus at 10 g/l and zinc at 0.038 g/l inhibit *E. coli* growth. A defined medium that contains the maximum non-inhibitive concentration of nutrients allows growth of *E. coli* to a cell density of about 15 g/l dcw (Shiloach and Fass, 2005).

Escherichia coli is a micro-organism that grows in both rich complex organic media and salt-based chemically defined media as long as carbon source is present . Besides an energy source, it requires nutrients for the biosynthesis of cellular matter, formation of products and maintenance so the content of the medium must supply the nutrients needed to accomplish this. The vital chemical elements needed for the cultivation of *E. coli* are hydrogen, carbon, nitrogen, oxygen, sodium, magnesium, phosphorus, potassium and calcium. These have specific functions during the bioactivity of cell growth and plasmid yield. Physiologically, hydrogen and oxygen form the basis of cellular water and with carbon as the main constituent of organic cell materials. Sodium, potassium, magnesium and calcium are cellular cations and cofactors for some enzymes. Phosphorous constitutes phospholipids, coenzymes and nucleotides in nucleic acids (Danquah and Forde, 2007).

Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, l-glutamine, l-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al*., 2006).

A study by Akhir *et al*., (2009) use chitin, peptone, yeast extract, sodium nitrate ($NaNO_3$) and *di*-potassium hydrogen phosphate (K_2HPO_4) as medium for the production of chitinase enzyme from shrimp waste. Vaidya et al., (2003) use ammonium sulfate $(NH_4)_2SO_4$, potassium *di*-hydrogen phosphate (KH_2PO_4), magnesium sulfate~heptahydrate ($MgSO_4 \cdot 7H_2O$), Tween 20, Yeast extract and Chitin for research statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans.*

2.3.2 Effect of Agitation Rate on Production of Chitinase Enzyme

The major roles of providing agitation in the fermenter are improved mixing, mass and heat transfer. Although increased agitation may provide increased mixing and mass transfer, it may also have many negative effects such as rupture of cells, change in morphological state, decrease in productivity, vacuolation and autolysis. Every fermentation has its own optimum agitator speed. This agitator speed will depend upon the resistance of the organism to shear, its morphological state, the nutrient composition, pH and many other conditions. It has been reported that agitation, pH, dissolved oxygen tension, polymer additives, surface active agents, growth rate and inoculum are the parameters that affect the productivity of a process. Among all these, agitator speed is the most critical parameter and plays a significant role in determining the productivity of the process. Agitator speed becomes important, as it will be one of the most critical parameters used for process scale-up (Felse and Panda, 2000).

Although increase in agitation and aeration may provide better mixing and mass transfer effects, excess in them might result in high shear stress and in turn lead to many negative effects, such as rupture of cells, vacuolation, autolysis and decrease in enzyme productivity. The optimal operation conditions of fermentation process also closely depend upon cell's morphological states, which in turn rely on the resistance of the organism to shear stress. It is generally believed that bacteria could resist more shear stress than fungi, however, little information is provided in the literature (Kao *et al*., 2006).

2.4 Response Surface Methodology (RSM)

Response surface methodology **(**RSM) is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables. In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables). Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables. If there is a curvature in the response, then a polynomial of higher degree, such as a second-order model, must be used to approximate the response, which is later analyzed to locate the optimum values of independent variables for the best response value. Using RSM requires special precautions to be taken to determine all critical variables sufficiently as well as not to work with too many variables over wide ranges (Aktasa *et al*., 2006).

The statistical software package, Design-Expert was used for regression analysis of the experimental data and also to plot the response surface graphs. Analysis of variance (ANOVA) was used to estimate the statistical parameters. To optimize the level of each factor for maximum response, point optimization process was employed. The combination of different optimized parameters, which gave maximum response of the maximum chitinase activity as it was tested experimentally to see the validity of the model (Valappil *et al.,* 2007).

The concept of RSM has eased process development and has been of significant use at industrial level. At a basic biological level, recent studies have indicated the use of RSM for analyzing effects of different factors on proteolytic activity and optimization of xylanase production. This study is an attempt to evaluate the effects of several factors on the production of an industrially important enzyme, chitinase (Nawani and Kapadnis, 2005).

Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions have successfully been used in the optimization of bio processes Gorret *et al*. have reported the maximization of the biomass content of oil palm (Elaeis guineensis) and analyzed the effects of nitrogen source, inoculum size and conditioned medium on biomass production using RSM. In another study, Almeida e Silva *et al*. used RSM in order to select nutrient levels for culturing Paecilomyces variotii in eucalyptus hemicellulosic hydrolyzate (Nikerel *et al*., 2006).

CHAPTER 3

METHODOLOGY

3.1 Overview of the methodology

Figure 3.1 show an overview process flow design for expression of recombinant chitinase. Basically, this study is divided into 3 steps to fulfill all the scopes of research. Firstly, study the effect of various medium. Secondly, effect of medium formulation and lastly, the optimization of medium formulation using RSM.

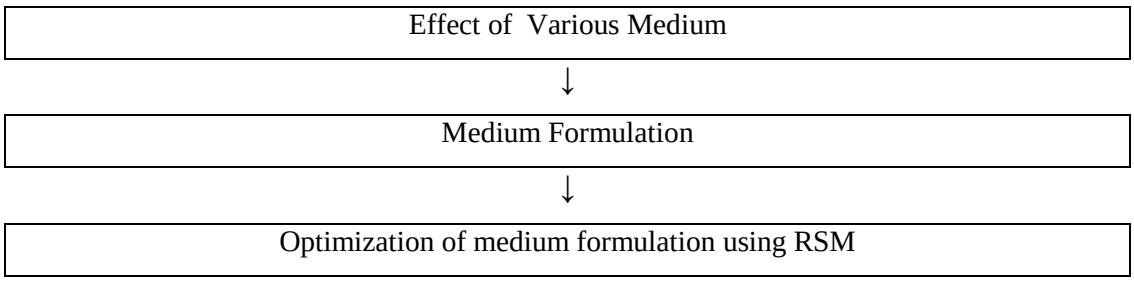


Figure 3.1: Overview process flow design for expression of recombinant Chitinase.

3.2 Colloidal Chitin Preparation

Colloidal chitin was prepared from purified chitin according to the method of Roberts and Selitrennikoff (1988) with a few modifications described as follows: 5 g of chitin flakes was added slowly into 900 mL of 37% hydrochloric acid (HCl) and stirred for 2 hour. The mixture was added to 500 mL of ice-cold 95 % ethanol under vigorous stirring for 30 min and kept overnight at 25 °C. The suspension was stored at -20 °C. When in need, 10 mL of the suspension is centrifuged and washed with 50 mL of 0.1M sodium phosphate buffer until colloidal chitin become neutral pH 7.0. The solution has about 10 g/L colloidal chitin solution.

3.3 Preparation of 0.2 M Sodium Phosphate Buffer (pH 6.5)

First, 13.9 gram of sodium phosphate monobasic is weighed and poured into 500 mL distilled water to make a 0.2M monobasic sodium phosphate. Then, 14.2 gram of sodium phosphate dibasic also weighed and poured into 500 mL distilled water to make a 0.2M dibasic sodium phosphate. To make 0.2M sodium phosphate buffer with pH 6.5, 205.5 mL of monobasic solution is mixed with 94.5 mL from dibasic solution. Monobasic solution is added to make the solution more acidic while dibasic solution was added to make the solution alkaline. The stock was stored at 4^{o}C.

3.4 Cell Cultivation

The medium uses are Luria Bertani (LB), Terrific Broth (TB), Superbroth (SB), Super Optimal Broth (SOB) and 2x YT.

1. LB: 10 g/l tryptone, 5g/l yeast, 5 g/l NaCl
2. TB: 12 g/l tryptone, 24 g/l yeast, 9.2 g/l KH_2PO_4, 2.2 g/l K_2PO_4
3. SB: 35 g/l tryptone, 20 g/l yeast, 5 g/l NaCl
4. SOB: 20 g/l tryptone, 5 g/l yeast, 10 g/l NaCl, 2.5 g/l KCl, 10 g/l $MgCl_2$, 10 g/l $MgSO_4$
5. 2x YT: 16 g/l tryptone, 10 g/l yeast, 5 g/l NaCl

Recombinant cells were cultivated in each medium. Addition of 100 μg/mL ampicilin as antibiotic will make the medium even more stabilize. The culture is growth in an incubator shaker at temperature 37 °C and 200 rpm rotational speed for 16 to 18 hours duration.

3.5 Culture Medium

The ratio of the culture and medium is 1 mL of culture to 50 mL of medium (1:50). The medium culture is growth in 250 mL shake flask at 200 rpm and temperature 37 °C. Every one hour, value of OD_{600} is check until its fall in the range of 0.4-0.5. Next is induction with IPTG to a final concentration of 1.0 mM and incubate at 25°C, 200 rpm Then, the culture is ready to undergo enzyme assay to analyze the chitinase activity, U/mL using dinitrosalicylic acid (DNS) method.

3.6 Preparation of Enzyme

Firstly, 50 mL of culture broth was harvested by centrifugation under condition 8000 rpm at temperature 4 °C for 10 minutes by using weighed centrifuge tube. The supernatant is discarded leaving behind the pellet and being washed by using 5 mL of 0.2 M sodium phosphate buffer to resuspendent gently. Centrifuge again at 8000 rpm for 10 minutes. The pellet resuspended with Bug Buster Extraction Reagent solution (2.5 mL Bug Buster per 50 mL cultures). Phenylmethylsulfonylfluoride (PMSF) was added as protease inhibitor which contains 8 μL of 10 mM PMSF per 50 mL cultures. The processes continued using ultrasonic for 10 minutes. The cell suspensions were incubated on a shaking platform at slow setting for 10 to 20 minutes in room temperature condition. The sample was centrifuged to remove the insoluble cell debris at 8000 rpm for 20 minutes at 4 °C.

3.7 Chitinase Enzyme Assay

Measurement of chitinase activity was done using a dinitrosalicyclic acid (DNS) method by Miller (1959). Preparation of DNS reagent: Sixteen gram dinitro salicylic acid (DNS) powder dissolved in 200 mL sodium hydroxide (NaOH) with concentration 2 mol/L. Potassium sodium tartarate weighed 300 g dissolved in 500 mL distilled water. Both chemical is mix and stir together for 30 minutes and distilled water added until the solution become 1 L. One mL of the crude enzyme was mixed with one mL of 0.2 M sodium phosphate buffer containing 10% colloidal chitin. The mixture incubated at 50 °C in water bath for 1 hour. Next, the reaction then stopped by 1 mL of 1% NaOH followed by boiling at 100 °C within 5 minutes. Then, the mixture was centrifuge at 5000 rpm for 10 minutes. One milliliter of supernatant collected and then 1 mL of DNS reagent (16 g DNS, 200 ml of 2M NaOH, 300 g K-Na-tartrate and 500 ml distilled water) added in before boiling it at 1000 °C for 10 minutes. The supernatant was subjected to spectrophotometric measurement at 535 nm. One unit of chitinase activity (U) was defined as the amount of enzyme that liberate 1 μmol of *N*-acetamino-β-D-glucose (GlcNAc) per minute at pH 5.4 and 50 °C (Ramirez et al., 2003).

3.8 Medium Formulation

The best medium, gives the highest chitinase activity in the *E. coli*, is chosen to study the medium formulation. The LB medium has three components which is sodium chloride (NaCl), yeast extract and tryptone. There will be three formulations, each for the component. NaCl formulation is to study the medium with variable composition of NaCl ranging within 1, 3, 5, 7 and 9 g/L. Yeast extract formulation is also to study the medium with variable composition of yeast extract ranging within 1, 3, 5, 7 and 9 g/L. Lastly, tryptone formulation is to study the medium with variable composition of tryptone ranging within 6, 8, 10, 12 and 14 g/L. This method will determine which value will be taken to be set as low and high value in the response surface methodology (RSM).

3.9 Optimization using Response Surface Methodology (RSM)

Software named Design Expert from company State-Ease Inc, Statistic Made Easy, Minneapolis, MN, USA was used for optimization under RSM. Central Composite Design (CCD) was used to optimize the levels of the significant variables. CCD is a design that contains an imbedded factorial or fractional factorial design with center points that is augmented with a group of `star points' that allow estimation of curvature. A CCD matrix was developed for each isolate depending on the number of factors considered for optimization. Each numeric factor is varied over 5 levels: $\pm \alpha$ (axial points), ± 1 (factorial points) and the center-point.

From the medium formulation method, the low and high value of the composition has been set. The mathematical interaction between parameters was studied. Optimization of culture conditions made up for the design of a 2^2 factorial. The total trial experiment given is 17 including 3 centre points. Table 3.1 shows the parameter and the centre points of the optimization.

Table 3.1: Optimization table of level study of medium formulation for further improvement of expression recombinant chitinase in *E. coli.*

Factor	Unit	Low level	Centre point	High level
Sodium Chloride	g/L	3	5	7
Yeast Extract	g/L	1.5	3	4.5
Tryptone	g/L	10	12	14

CHAPTER 4

RESULT AND DISCUSSION

4.1 The Effect of Various Medium on Production of Chitinase

The effect of various medium was carried out with different variables which are LB, TB, SB, SOB and 2x YT. During the fermentation process, other parameters that can affect the production of chitinase are kept constant throughout the research. Agitation rate was set at 200 rpm, temperature at 37°C, incubation time 17 hour, and inducing time for 6 hour at 25°C. Figure 4.1 show the result of various medium that affected the production of chitinase.

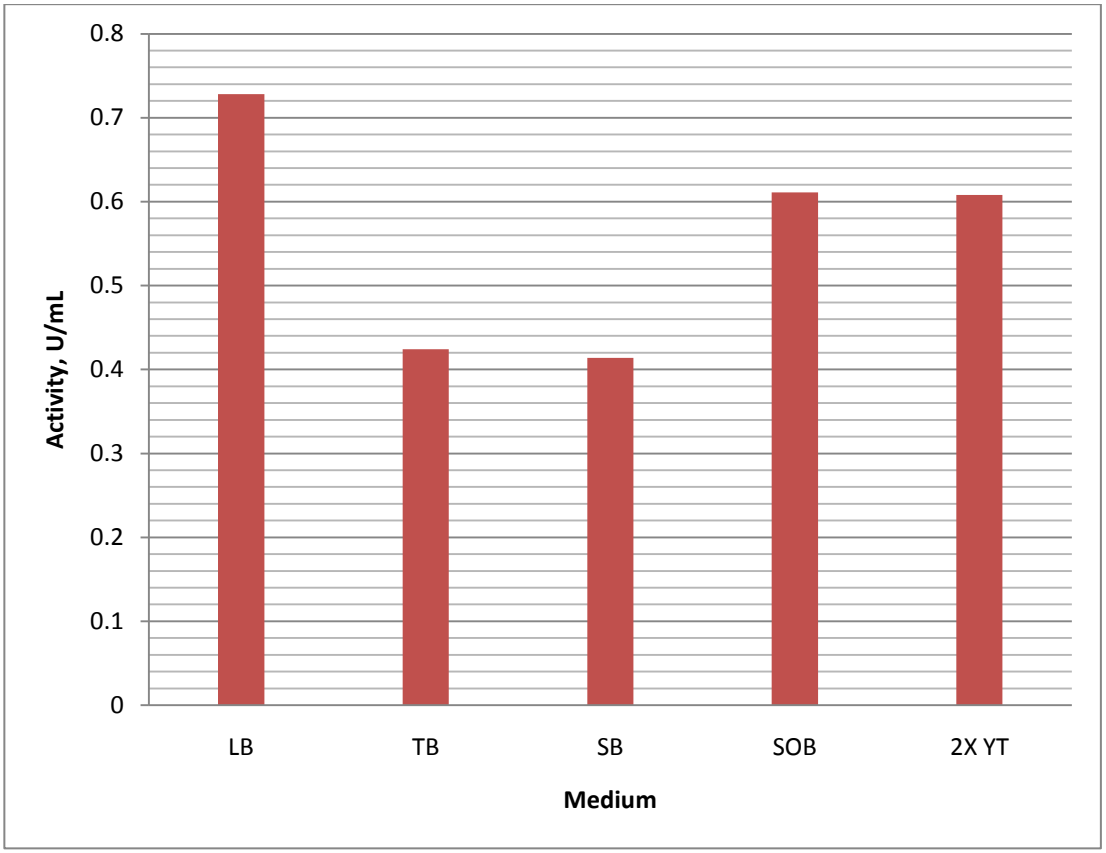


Figure 4.1: The effect of various medium.

Based on observation and value in Figure 4.1, the results showed that, within the 5 medium, LB medium gives the highest activity chitinase yields with 0.728 U/ml. SB medium yields the lowest enzyme activity with the value of 0.414 U/ml. Sodium chloride ionizes in the aqueous solution, forming Na+ and Cl− ions. The osmolarity of sodium chloride is double the osmolarity of sucrose, if molar concentrations of both solutes are equal. The higher osmolarity of sodium chloride contributes to its more severe growth restrictive effect (Kawarai et al., 2009). The composition of sodium chloride in LB medium contains higher osmolarity compared to other medium. Media containing yeast extract and hydrolysed protein are often used because they are relatively simple to prepare and generally lead to high cell densities. Meat extracts are also rich sources of nutrients for fermentation process (Danquah and Forde, 2007). LB is chosen as the best medium and will undergo medium formulation for each of its component composition.

4.2 The Effect of Medium Formulation on Production of Chitinase Using Conventional Method

4.2.1 Sodium Chloride (NaCl) Formulation

Medium is prepared with variable value of sodium chloride (NaCl) composition. Yeast extract and tryptone composition is kept constant at 5 and 10 g/l respectively, while the NaCl composition is manipulated with the value of 1, 3, 5, 7 and 9 g/l. Figure 4.2 show the effect of NaCl formulation.

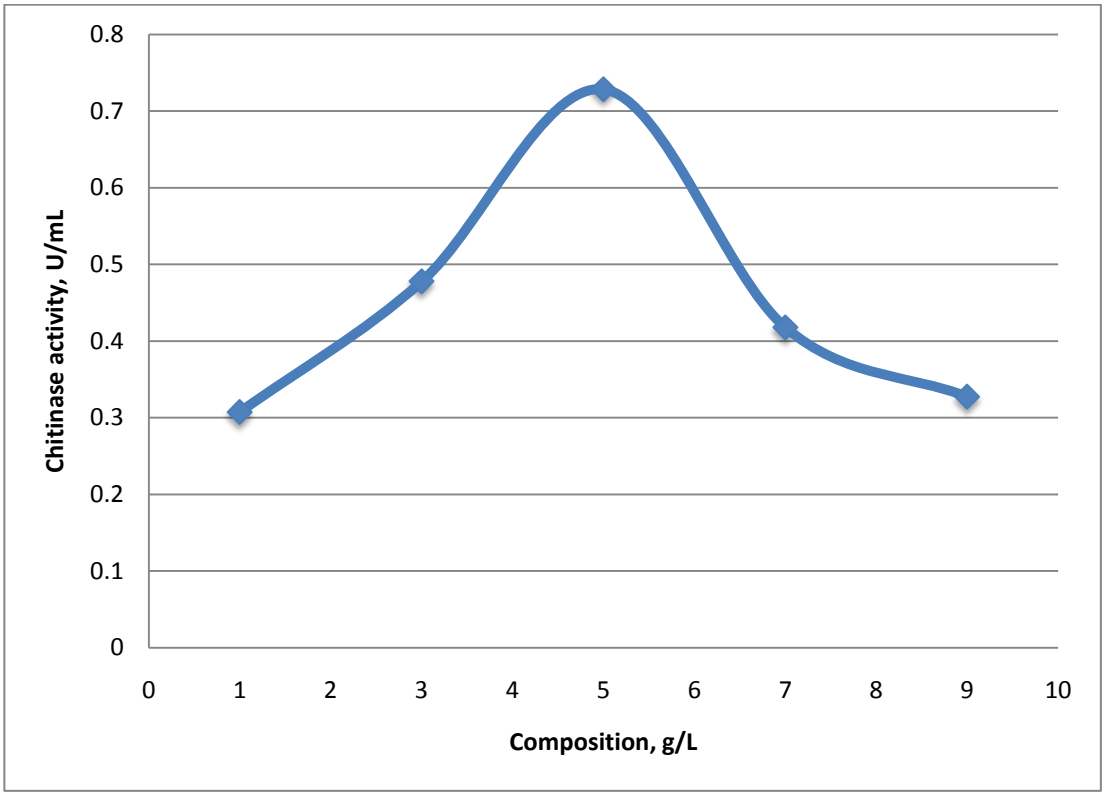


Figure 4.2: The effect of NaCl formulation.

From the Figure 4.2, we can see the pattern which is the activity value keep increasing from 1 g/l until composition 5 g/l and then the value keep decreasing. The highest value of activity is at 5 g/l with 0.728 U/ml while the lowest activity at 1 g/l with 0.308 U/ml. Hajmer *et al*., (2004) stated that the sodium chloride composition for LB medium is best at 5g/l, the original value itself. The best value, 5 g/l is taken and use for further step of this study, the yeast extract formulation. The best range taken for optimization is 3 g/l to 7 g/l.

4.2.2 Yeast Extract Formulation

Medium is prepared with variable value of yeast extract composition. Sodium chloride and tryptone composition is kept constant at 5 and 10 g/l respectively, while the yeast extract composition is manipulated with the value of 1, 3, 5, 7 and 9 g/l. Figure 4.3 show the effect of yeast extract formulation.

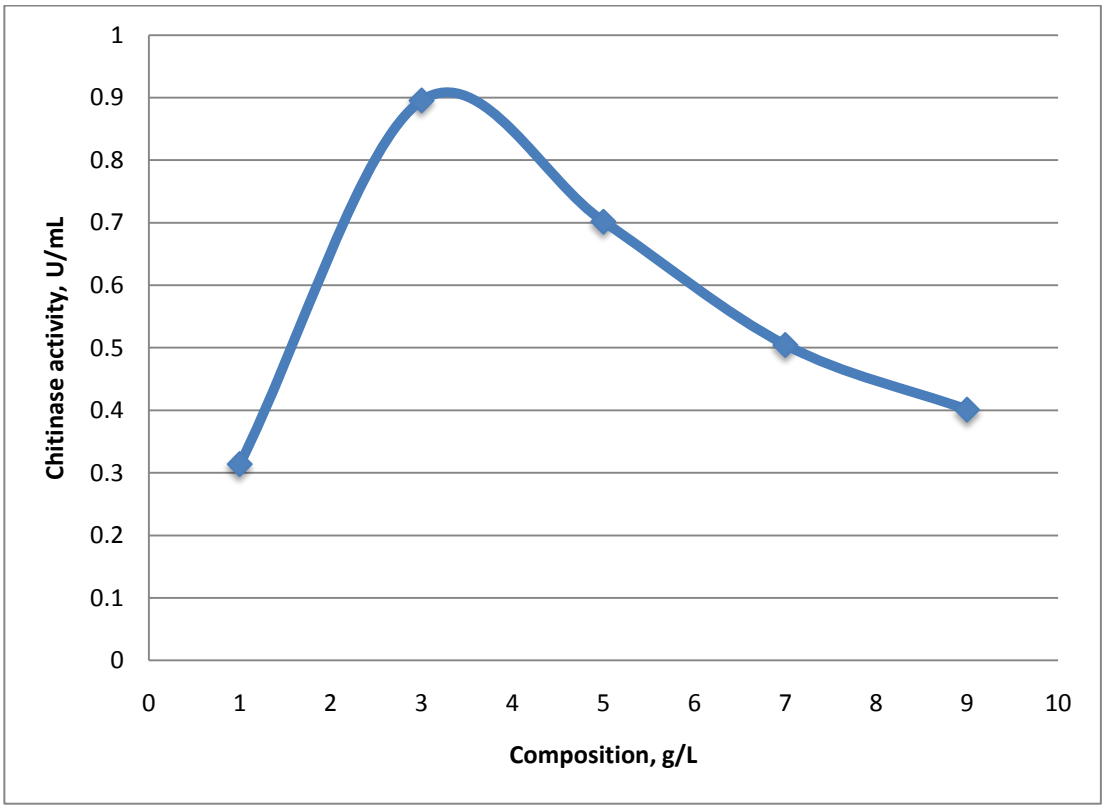


Figure 4.3: The effect of yeast extract formulation.

From the Figure 4.3, the pattern which is the activity value keep increasing from 1 g/l until composition 3 g/l and then the value keep decreasing. The highest value of activity is at 3 g/l with 0.895 U/ml while the lowest activity at 1 g/l with 0.314 U/ml. The best value, 3 g/l is taken and use for further step of this study, the tryptone formulation. The best range taken for optimization is 1.5 g/l to 4.5 g/l. The range shown is quite similar to Nawani and Kapadnis (2005) study when they use the range of 0.1 g/l to 0.4 g/l yeast extract.

4.2.3 Tryptone Formulation

Medium is prepared with variable value of tryptone composition. Sodium chloride and yeast extract composition is kept constant at 5 and 3 g/l respectively, while the tryptone composition is manipulated with the value of 6, 8, 10, 12 and 14 g/l. Figure 4.4 show the effect of tryptone formulation.

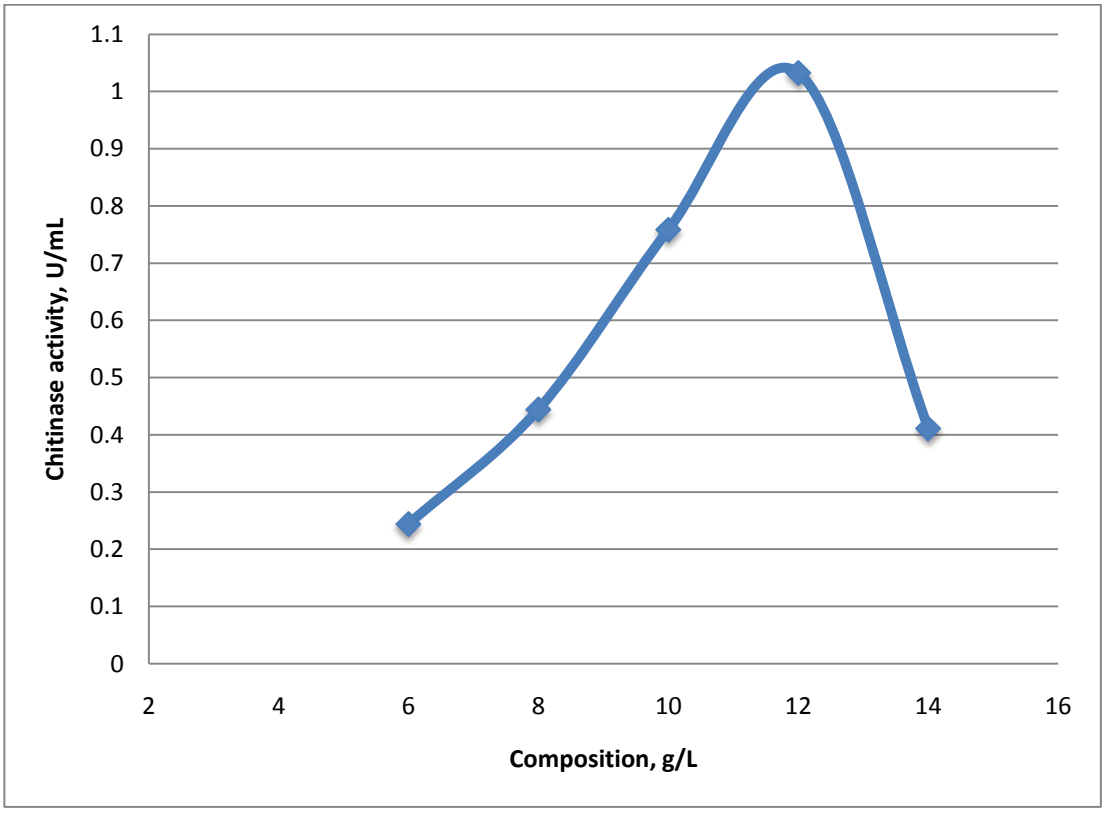


Figure 4.4: The effect of tryptone formulation.

Pattern form Figure 4.4 shown that the activity value keeps increasing from 6 g/l until composition 12 g/l and then the value keeps decreasing. The highest value of activity is at 12 g/l with 1.032 U/ml while the lowest activity at 1 g/l with 0.244 U/ml. The best value, 12 g/l is taken. The best range for optimization with low and high value has been set is 10 g/l to 14 g/l. The range shown is quite similar to Han *et al*., (2008) that use peptone with low value of 10 g/l and high value of 15 g/l. As peptone composition is quite similar to the tryptone.

4.3 Optimization of Medium Formulation on Chitinase Production using Response Surface Methodology (RSM)

Design Expert 6.0 software was used to identify the best composition for sodium chloride (NaCl), yeast extract and tryptone. A smaller and less time consuming experimental design, response surface methodology (RSM) could generally satisfy the optimization of many microbial processes. Table 4.1 shows the value from the conventional method that will be used in the software to continue for optimization process.

Table 4.1: Lower level and upper level of medium formulation.

Component	**Low Level**	**High Level**
Sodium chloride, X_1	3	7
Yeast extract, X_2	1.5	4.5
Tryptone, X_3	10	14

By using central composite design (CCD), the software came up with 17 run of experiment with 3 centre point. All 17 medium were prepared precisely according to the actual value as stated in Table 4.2. The result obtained after CCD were then analyzed by ANOVA, which has gave the following regression equation as:

$$\text{Log}_{10}\, Y = 0.16 - 0.059X_1 + 0.22X_2 + 1.026\text{x}10^{-3}X_3 - 0.29X_1^2 - 0.058X_2^2 - 0.12X_3^2 - 0.17X_1X_2 - 0.29X_1X_3 - 0.065X_2X_3$$

X_1 - Sodium chloride

X_2 - Yeast extract

X_3 - Tryptone

Summary in Table 4.2 showed the chitinase production for each individual standard order with the predicted responses and actual value. The results showed that chitinase yields varied within the range of 0.083 to 2.563 U/ml. The standard order number 14 gave the highest of chitinase activity which was 2.563 U/mL with composition of NaCl, yeast extract and tryptone were 5.00, 5.52 and 12.00 g/l respectively. On the other hand, conditions of standard order number 4 with composition of NaCl, yeast extract and tryptone were 3.00, 1.50 and 10.00 g/l respectively were observed that gave the lowest chitinase activity 0.083 U/mL.

Table 4.2: Summary of central composite design matrix.

Standard Order	Coded Value (Actual value)			Chitinase Activity (U/ml)	
	X_1 (g/l)	X_2 (g/l)	X_3 (g/l)	Actual	Predicted
1	7.00	1.50	10.00	0.843	0.963
2	3.00	4.50	14.00	1.837	1.947
3	3.00	4.50	10.00	0.865	0.848
4	3.00	1.50	10.00	0.083	0.168
5	7.00	4.50	10.00	0.912	0.985
6	3.00	1.50	14.00	0.623	0.541
7	7.00	4.50	14.00	0.264	0.170
8	5.00	3.00	8.64	0.682	0.663
9	8.36	3.00	12.00	0.15	0.091
10	5.00	0.48	12.00	0.368	0.426
11	5.00	3.00	12.00	1.847	1.737
12	5.00	3.00	15.36	0.611	0.643
13	1.64	3.00	12.00	0.321	0.262
14	5.00	5.52	12.00	2.563	2.518
15	5.00	3.00	12.00	1.22	1.292
16	7.00	1.50	14.00	0.227	0.228
17	5.00	3.00	12.00	1.333	1.367

The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2, adjusted R^2, predicted R^2 and ‘‘adequate precision’’. Table 4.3 shows the regression analysis which was analyzed by ANOVA for the production of recombinant chitinase in *E coli*. The R^2 is 0.9679 pointed to the value of coefficient regression. The adjusted R^2 is 0.9266 and predicted R^2 is 0.7673. All the value is over 0.85 which shows the model is significant and a good model. Therefore this study carried out adequate precision which is 16.032 and is reasonable for this study.

Table 4.3: ANOVA for response surface quadratic model.

Model Term	**Value**
R^2	0.9679
Adj R^2	0.9266
Pred R^2	0.7673
Adeq precision	16.032

Table 4.4 showed the ANOVA and regression analysis of the response for chitinase production. *P*-value is used to check the significance of each coefficient and it is necessary to understand the pattern of the interaction between the variable (Li *et al.,* 2007). When the *P*-value smaller than 0.05 there are the higher of significant of variable interaction. In this study the, *P*-value is 0.0002 that had been summarized in Table 4.4. The non significant of lack of fit which is 0.4419 is more than 0.05 which indicates the model is a good fit.

Table 4.4: ANOVA and regression analysis of the response for chitinase production.

Sources	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F-value**	***P*-value (Prob >F)**	**R^2**
Model	2.6085	9	0.010	23.46	0.0002	0.9679
Residual	0.0865	7	0.012	-	-	
Lack of Fit	0.0685	5	0.014	1.52	0.4419	
Pure Error	0.0180	2	$8.997x10^{-3}$	-	-	
Correlation Total	2.6950	16	-	-	-	

Table 4.9 shows the regression coefficient and *P*-value obtained from calculation by ANOVA. When the Prob>F value less than 0.05, the model in term X_2, X_1^2, X_3^2, X_1X_2 and X_1 X_3 are considered as significant except X_1, X^3, X_2^2 and X_2X_3.

Table 4.5: Regression coefficients and *P*-value calculated from the model.

Variables	**Coefficient**	***P*-value (Prob > F)**
Offset	0.16	-
X_1	-0.059	0.0492
X_2	0.22	0.0002
X_3	$0.026x10^{-3}$	0.9737
X_1^2	-0.029	< 0.0001
X_2^2	-0.058	0.1220
X_3^2	-0.12	0.0081
X_1X_2	-0.17	0.0031
X_1X_3	-0.29	0.0002
X_2X_3	-0.065	0.1441

The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the responses and the experimental levels of each factor used in the design. From the result obtained by analyzing through ANOVA, the three dimension response surface curve was plotted as in Figure 4.5. This figure explained about the interaction between the sodium chloride and yeast extract.

Figure 4.5 shows the 3D response surface plot, sodium chloride (NaCl) versus yeast extract. From the figure, when yeast extract is constant at 1.5 g/l, the surface shows increase of activity when the composition of NaCl increases, however the activity started to decrease after 5 g/l of NaCl. The highest of the activity shown was in the area 5 g/l and is the optimum formulation for the NaCl while the lowest activity was at 3 g/l. Gohel *et al*., (2006) stated that the rest of the components chitin, peptone, yeast extract, urea, NH_4NO_3, NaCl, $CaCl_2$, KBr, $MgSO_4 \cdot 7H_2O$, KNO_3 and KH_2PO_4 showed confidence level at or above 95% and were considered to be significant. These components can be used for high chitinase production.

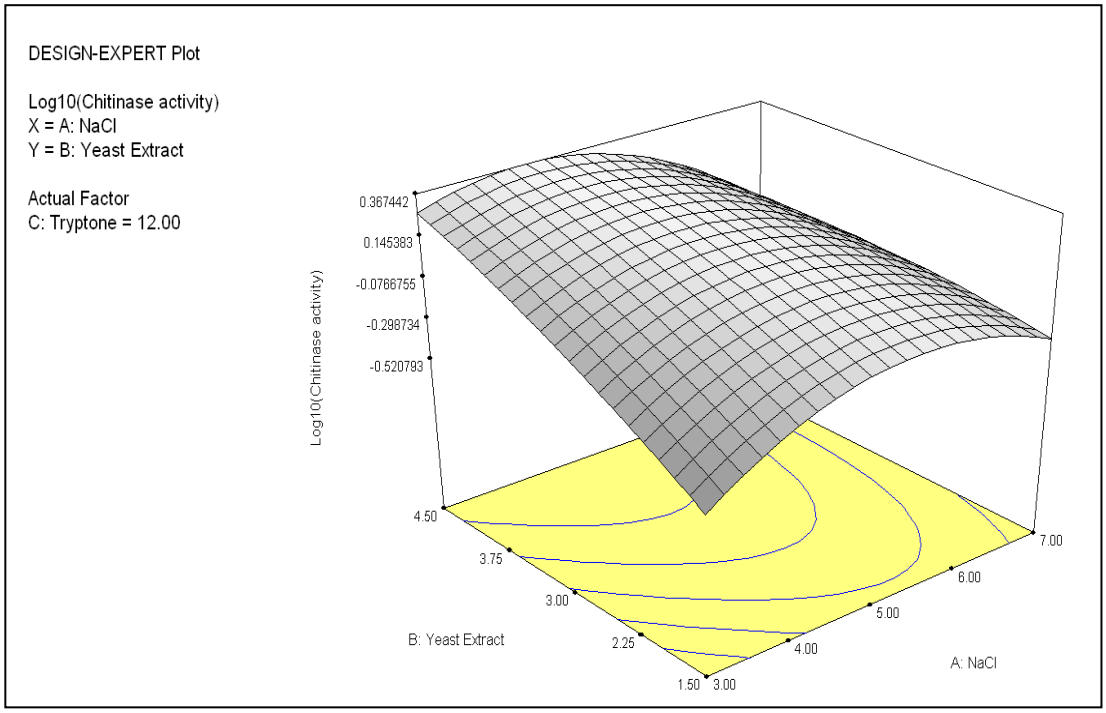


Figure 4.5: Response surface plot of recombinant chitinase production: yeast extract versus sodium chloride.

Figure 4.6 also shows the 3D response surface plot, but with different component, tryptone versus yeast extract. From the figure, when tryptone was constant at 10.0 g/l, the surface shows increase of enzyme activity when the composition of yeast extract increases. The highest of the activity shown was in the area 4.5 g/l and is the optimum formulation for the yeast extract while the lowest activity was at 1.5 g/l. Gohel *et al*., (2006) stated that the rest of the components chitin, peptone, yeast extract, urea, NH_4NO_3, NaCl, $CaCl_2$, KBr, $MgSO_4{\cdot}7H_2O$, KNO_3 and KH_2PO_4 showed confidence level at or above 95% and were considered to be significant. All these components are contain in LB medium and can be used for high chitinase production.

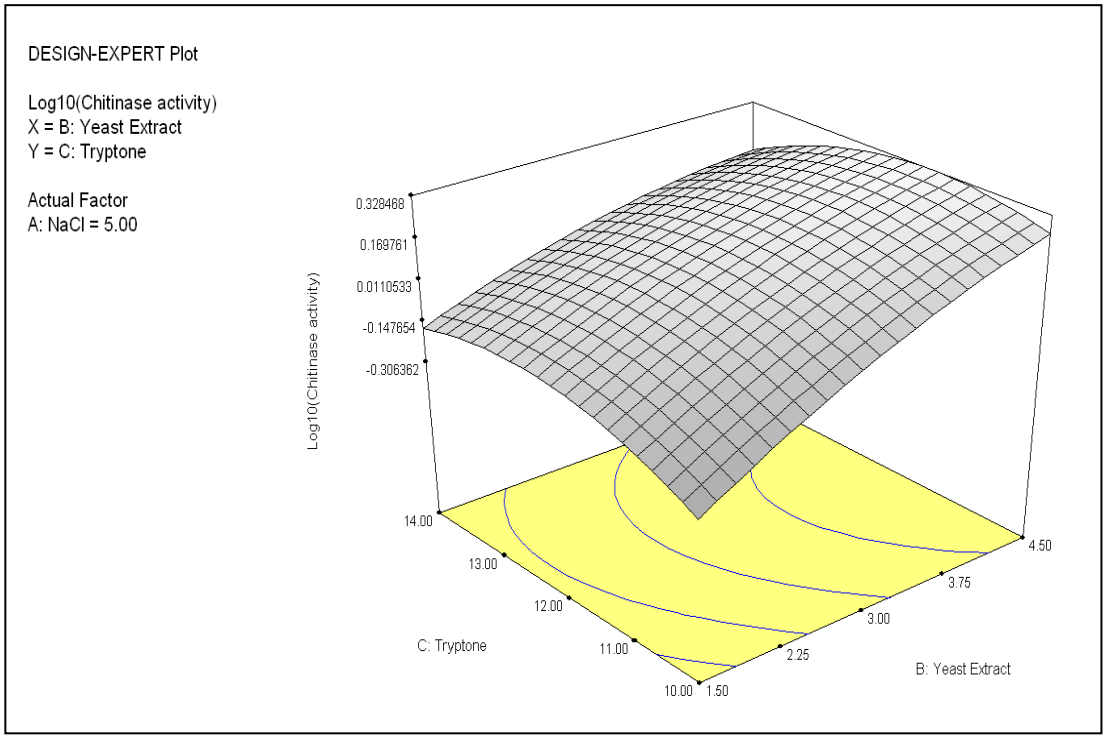


Figure 4.6: Response surface plot of recombinant chitinase production: tryptone versus yeast extract.

Figure 4.7 shows the 3D response surface plot of, tryptone versus NaCl. From the figure, when NaCl was constant at 3.0 g/l, the surface shows increase of enzyme activity when the composition of tryptone increases. The highest of the activity shown was in the area 13.0 g/l to 14.0 g/l and is the optimum formulation for the yeast extract while the lowest activity was at 10.0 g/l. Gohel *et al*., (2006) stated that the rest of the components chitin, peptone, yeast extract, urea, NH_4NO_3, NaCl, $CaCl_2$, KBr, $MgSO_4{\cdot}7H_2O$, KNO_3 and KH_2PO_4 showed confidence level at or above 95% and were considered to be significant. All these components are contain in LB medium and can be used for high chitinase production.

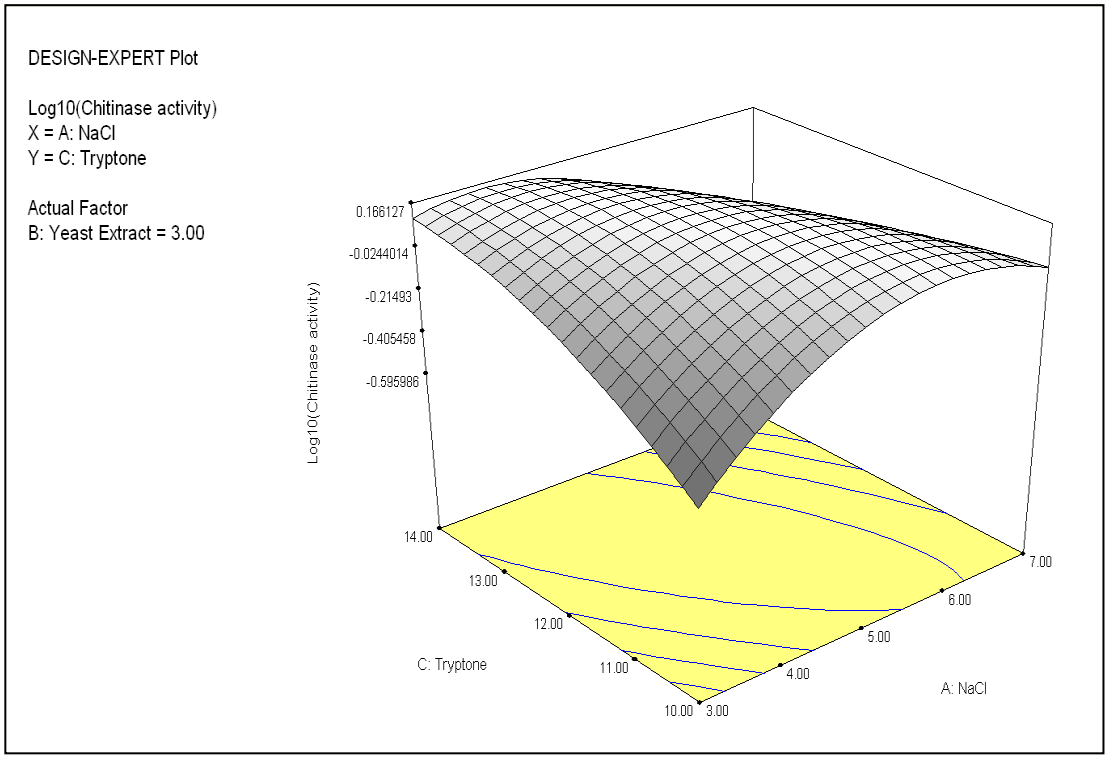


Figure 4.7: Response surface plot of recombinant chitinase production: tryptone versus NaCl.

Table 4.6 shows the summary of the optimized medium for production of chitinase. The result obtained before and after optimization is analyzed. The software combined the optimized variable to give the best medium composition which should yield the highest production of chitinase with 2.409 U/ml of chitinase activity. The composition is 3.63 g/l of sodium chloride, 4.50 g/l of yeast extract and 13.11 of tryptone.

Verification experiment was successfully accomplished by using the optimization condition and gave the production of chitinase of 2.291 U/ml, which is close to the predicted response of 2.409 U/ml. This result corroborated the predicted values and effectiveness of the model formed. The result shows an increasing enzyme activity compared to the activity before the optimization which is 0.411 U/ml. The optimization also shows that most of the component compositions were reduced to obtain the highest chitinase production thus saving the cost. Yeast extract usage reduced from 5.0 g/l to 4.5 g/l and tryptone from 14.0 g/l to 13.11 g/l. The determination of predicted and experimental enzyme activity that was maximized by the optimized culture conditions is detailed in Table 4.6.

Table 4.6: Summary of the Optimized Medium for Production of Chitinase.

Parameters	**After Optimization**			**Before Optimization**	
	Value (g/l)	**Chitinase Activity (U/ml)**		**Value (g/l)**	**Chitinase Activity (U/ml)**
		Predicted	**Actual**		
Sodium chloride	3.63	2.409	2.291	3.00	0.411
Yeast extract	4.50			5.00	
Tryptone	13.11			14.00	

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The production of chitinase from recombinant chitinase in *E. coli* was successfully carried out. LB medium gives the best result for enzyme activity. Medium formulation is undergone for determining of low and high level to be used in response surface methodology (RSM). The optimization of the cultural medium to improve the expression and production of chitinase was achieved by using RSM. The optimum medium culture conditions are 3.63 g/l of sodium chloride, 4.50 g/l of yeast extract and 13.11 g/l of tryptone. The maximum value achieved after the optimization is 2.291 U/ml. This condition gave increment enzyme activity compared before the optimization which produced 0.150 U/ml of chitinase activity.

5.2 Recommendation

In order to improve this research, there are several things should be stress out in the future. Firstly is using low temperature for inducing process such as at 18°C. Robert *et al*., (2004) reported that low temperature for inducing the *E. coli* will enhance the expression system rapidly. The optimum temperature stated by Robert *et al*., (2004) is at 18°C. Thus, decreasing the inducing temperature may produce more chitinase, and will help the research to get more accurate result.

Furthermore, this process is actually an energy consuming process and it is recommended to use simultaneous hydrolysis and fermentation. To improve the enzymatic hydrolysis, the suitable method must be use to avoid denaturing of enzyme where it can affect the percentage of yield production. During the expression, the chemical cell disruption method is suggested to break down the cell wall of bacteria comparing using sonicator which is the mechanical cell disruption that may occur the denaturing of protein by heat.

For a better and accurate result, we should try to induce the IPTG at lower concentration such as 0.1mM. The final concentration of an inducer is actually affecting the production of chitinase (Miriyam *et al*., 2005). Optimum inducer concentration will give better chitinase production thus giving a better accurate result for research in future.

Last but not least for further improvement, the suggestion of the upscale production of chitinase using 2 or 5 L of fermentor. These will help the research for the industrial field beside to increase the product in large scale production.

REFERENCES

Akhir, S. M., Abd-Aziz, S., Salleh, M. M., Rahman, R. A., Illias, R. M. and Hassan, M. A., Medium Optimization of Chitinase Enzyme Production from Shrimp Waste using Bacillus licheniformis TH-1 by Response Surface Method, Biotechnology 8 (1) (2009), pages 120-125

Aktasa, N., Boyaci, I. H., Mutlu, M. and Tanyolac, A., Optimization of lactose utilization in deproteinated whey by *Kluyveromyces marxianus* using response surface methodology (RSM), Bioresource Technology 97 (2006), pages 2252–2259

Badariottia, F., Thuaub, R., Lelonga, C., Dubosa, M. P. and Favrela, P., Characterization of an atypical family 18 chitinase from the oyster *Crassostrea gigas*: Evidence for a role in early development and immunity Developmental and Comparative Immunology 31 (2007), pages 559–570

Danquah, M. K and Forde, G. M., Growth Medium Selection and Its Economic Impact on Plasmid DNA Production, JOURNAL OF BIOSCIENCE AND BIOENGINEERING Vol. 104, No. 6 (2007), pages 490–497

Fan, Y., Zhang, Y., Yang, X., Pei, X., Guo, S., and Pei Y., Expression of a *Beauveria bassiana* chitinase (Bbchit1) in *Escherichia coli* and *Pichia pastoris*, Protein Expression and Purification 56 (2007), pages 93–99

Felse, P. A. and Panda, T., Submerged culture production of chitinase by *Trichoderma harzianum* in stirred tank bioreactors – the influence of agitator speed, Biochemical Engineering Journal 4 (2000), pages 115–120

Hajmeer, M. N., Marsden, J. L., Fung, D. Y. C. and Kemp, G. K., Water, sodium chloride and acidified sodium chlorite effects on *Escherichia coli* O157:H7 and *Staphylococcus aureus* on beef briskets, Meat Science 68 (2004), pages 277–283

Han, Y., Li, Z., Miao, X. and Zhang, F., Statistical optimization of medium components to improve the chitinase activity of *Streptomyces sp*. Da11 associated with the South China Sea sponge *Craniella australiensis*, Process Biochemistry 43 (2008), pages 1088–1093

Kao, P. M., Chen C. I., Huanga, S. C., Chang, Y. C., Tsai, P. J. and Liu, Y. C., Effects of shear stress and mass transfer on chitinase production by *Paenibacillus* sp. CHE-N1, Biochemical Engineering Journal 34 (2007), pages 172–178

Kawarai, T., Furukawa, S., Narisawa, N., Hagiwara, C., Ogihara, H. and Yamasaki, M., Biofilm formation by *Escherichia coli* in hypertonic sucrose media, Journal of Bioscience and Bioengineering VOL. 107 No. 6 (2009), pages 630–635

Kirubakaran, S. I. and Sakthivel, N., Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*, Protein Expression and Purification 52 (2007), pages 159–166

Kuttiyawong, K., Nakapong, S. and Pichyangkura, R., The dual exo/endo-type mode and the effect of ionic strength on the mode of catalysis of chitinase 60 (CHI60) from *Serratia sp*. TU09 and its mutants, Carbohydrate Research 343 (2008), pages 2754–2762

Larsen, M. W., Bornscheuer, U. T. and Hult, K., Expression of *Candida antarctica* lipase B in *Pichia pastoris* and various *Escherichia coli* systems, Protein Expression and Purification 62 (2008), pages 90–97

Liu, B. L., Kao, P. M., Tzeng Y. M. and Feng, K. C., Production of chitinase from *Verticillium lecanii* F091 using submerged fermentation, Enzyme and Microbial Technology 33 (2003), pages 410–415

Nampoothiri, K. M., Baiju T.V., Sandhya, C., Sabu, A., Szakacs, G. and Pandey, A., Process optimization for antifungal chitinase production by *Trichoderma harzianum*, Process Biochemistry 39 (2004), pages 1583–1590

Nawani, N.N. and Kapadnis, B.P., Optimization of chitinase production using statistics based experimental designs, Process Biochemistry 40 (2005), pages 651–660

Nikerel, E., Toksoy, E., Kırdar, B. and Yıldırım, R., Optimization of medium composition for biomass production of recombinant *Escherichia coli* cells using response surface methodology, Biochemical Engineering Journal 32 (2006), pages 1-6

Nikerel, E., Toksoy, E., Kırdar, B. and Yıldırım, R., Optimizing medium composition for TaqI endonuclease production by recombinant *Escherichia coli* cells using response surface methodology, Process Biochemistry 40 (2005), pages 1633–1639

Pan, H., Xiea, Z. Baob, W. and Zhanga, J., Optimization of culture conditions to enhance *cis*-epoxysuccinate hydrolase production in *Escherichia coli* by response surface methodology, Biochemical Engineering Journal 42 (2008), pages 133–138

Patidar, P., Agrawal, D., Banerjee, T. and Patil, S., Optimisation of process parameters for chitinase production by soil isolates of *Penicillium chrysogenum* under solid substrate fermentation, Process Biochemistry 40 (2005), pages 2962–2967

Reetarani S. Patil, Ghormade, V. and Deshpande, M.V. (2000). Chitinolytic Enzymes: An Exploration. *Enzyme and Microbial Technology*. 26: 473-483

Seto, M., Ogawa, T., Kodama, K., Muramoto, K., Kanayama, Y., Sakai, Y., Chijiwa, T. and Ohno, M., A novel recombinant system for functional expression of myonecrotic snake phospholipase A2 in *Escherichia coli* using a new fusion affinity tag, Protein Expression and Purification 58 (2008), pages 194–202

Shiloach, J., and Fass, R., Growing *E. coli* to high cell density—A historical perspective on method development, Biotechnology Advances 23 (2005), pages 345–357

Sørensen, H. P. and Mortensen, K. K., Advanced genetic strategies for recombinant protein expression in *Escherichia coli*, Journal of Biotechnology 115 (2005), pages 113–128

Stephan, M., Beshay, U., Friehs, K. and Flaschel, E., Influence of medium composition on growth behaviour of *Dictyostelium discoideum* for cultivation on axenic media, Process Biochemistry 39 (2003), pages 333_ 343

Suresh, P. V. and Chandrasekaran, M., Impact of process parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation, Process Biochemistry 34 (1999), pages 257–267

Tokuyasu, K., Kaneko, S., Hayashi, K. and Mori, Y., Production of a recombinant chitin deacetylase in the culture medium of *Escherichia coli* cells, FEBS Letters 458 (1999), pages 23-26

Vaidya, R., Vyas, P. and Chhatpar, H.S., Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*, Enzyme and Microbial Technology 33 (2003), pages 92–96

Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P., Jaruseranee, N. and Buranabanyat, B., Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems, Journal of Biotechnology 133 (2008), pages 50–57

Yong, T., Hong, J., Zhang-Fu, L., Li, Z., Xiu-Qiong, D., Ke, T. and Shi-Gui, L., Cloning and Expression of a Chitinase Gene from *Sanguibacter* sp. C4, *Acta Genetica Sinica*, 33 (11) (2006), pages 1037–1046

APPENDIX A

Enzymatic Assay of Chitinase

Colorimetric assay;

Standard Curve:

A standard curve is prepared by pipetting (in milliliters) the following reagents into suitable containers:

Appendix A.1: Data for standard calibration curve of NAG

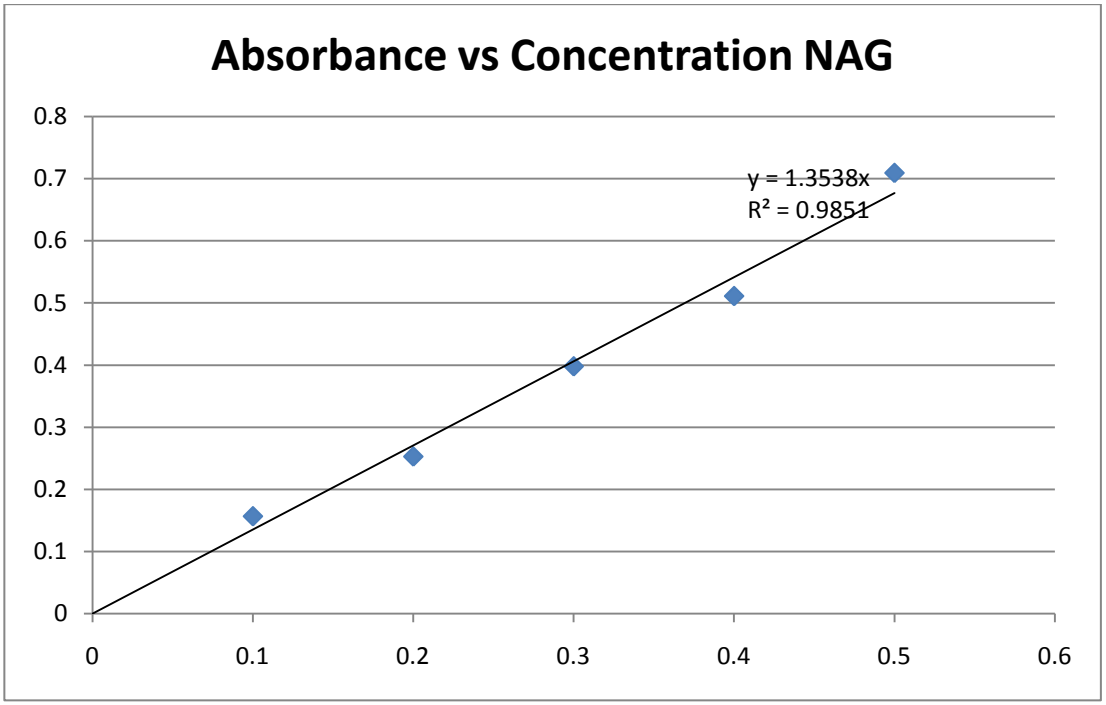
	Std 1	Std 2	Std 3	Std 4	Std 5	Std Blank
Reagent H (NAG Std Soln)	0.1	0.2	0.3	0.4	0.5	0.0
Deionized Water	2.9	2.8	2.7	2.6	2.5	3.0
Reagent E (Clr Rgt Soln)	1.5	1.5	1.5	1.5	1.5	1.5

Calculations;

Standard Curve:

D A540 nm Standard = A540 nm Std - A540 nm Std Blank

Plot the DA540nm of the standards versus milligrams of NAG.



Appendix A.2: Graph of standard curve NAG

The graph above was constructed based on the data obtained after the standard NAG was tested by using DNS method. This graph was important in order to find the optical density of NAG in every sample being tested. This preparation is important in order to find the optical density of tested. The gradient of the trendline was used in calculation of chitinase activity in following equation.

$$\text{Enzyme Activity (U/mL)} = \frac{\text{OD x 1000}}{\text{Slope x MW}}$$

where; OD : optical density at a wavelength of 535 nm

Slope : 1.353 (from standard curve Appendix A.2)

MW : molecular weight of NAG, 221.21 g/mole