OPTIMIZATION OF INDUCERS ON EXPRESSION OF RECOMBINANT CHITINASE IN *Escherichia coli* USING RESPONSE SURFACE METHODOLOGY.

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I declare that this thesis entitled "Optimization of Inducers on Expression of Recombinant Chitinase in *Escherichia coli* using Response Surface Methodology" 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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Date : 30 April 2009

To my beloved mother and father

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ABSTRACT

The function of chitinase as a chitin digestive enzyme has attracted the attention as one of the potential materials for control of phytopathogenic fungi and insect pests. However, chitinase is uneconomic for commercialized due to the high cost in its production. The development in recombinant DNA technology has helped to reduce chitinase production cost and enhance its production. The aim of this study is to determine the optimum conditions toward the production of recombinant chitinase in *Escherichia coli* by manipulating the concentration of inducers (IPTG and lactose). The best concentration of IPTG and lactose as inducers were determined by using the conventional method. Results from conventional method were used for further improvement of optimization by using Response Surface Methodology (RSM). In Response Surface Methodology, the optimization of expression was carried out based on the Central Composite Design (CCD). The effect of the inducers concentration toward the expression level of the recombinant chitinase production were analyzed and optimum values of tested variables for the production of chitinase were 1.19 mM IPTG and 0.98% lactose. Response surface analysis revealed that 0.513 U/ml of chitinase activity was achieved by using the optimized condition. This improved about 13.2-fold compare to initial experiment which produced 0.039 U/ml. This showed that combination between IPTG and lactose has increased the production of recombinant chitinase.

ABSTRAK

Fungsi kitinase yang bertindak sebagai enzim pengurai kitin telah menjadikannya sebagai bahan yang berpotensi untuk mengawal kulat fitopatogenik dan serangga perosak. Walau bagaimanapun, kitinise dikatakan tidak ekonomik untuk dikomersilkan kerana kos penghasilannya yang tinggi. Pembangunan di dalam bidang teknologi rekombinan DNA telah membantu mengurangkan kos penghasilan kitinase dan meningkatkan produktivitinya. Tujuan kajian ini dilakukan adalah untuk mengenal pasti keadaan yang optimum di dalam penghasilan kitinase rekombinan di dalam *Escherichia coli* dengan memanipulasikan kepekatan pengaruh (IPTG dan laktosa). Kepekatan yang terbaik untuk IPTG dan laktosa ditentukan dengan menggunakan kaedah konvensional. Keputusan daripada kaedah konvensional ini kemudiannya digunakan di dalam pengoptimuman yang seterusnya dengan mengunakan kaedah tindak balas permukaan (RSM). Di dalam kaedah tindak balas permukaan, rekabentuk komposit pusat (CCD) digunakan untuk mengoptimumkan pengekspresan. Kesan kepekatan pengaruh terhadap tahap pengekspresan kitinase rekombinan dianalisis dan nilai optimum bagi pemboleh ubah yang diuji adalah 1.19 mM IPTG dan 1.0% laktosa. Analisis gerak balas menunjukkan sebanyak 0.513 U/ml aktiviti kitinase telah dicapai di dalam keadaan yang optimum. Ini telah meningkat sebanyak 13.2-kali ganda jika dibandingkan dengan kajian awal yang hanya menghasilkan sebanyak 0.039 U/ml aktiviti kitinase. Ini menunjukkan penggabungan IPTG dan laktosa telah meningkatkan penghasilan kitinase rekombinan.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
CCD	-	Central composite design
g	-	Gram
g/L	-	Gram per litre
hr	-	Hour
IPTG	-	Isopropl-β-D-thiogalactoside
L	-	Litre
M	-	Molar
mg	-	Miligram
min	-	Minutes
ml	-	Mililitre
mM	-	Milimolar
nm	-	Nanometer
OD	-	Optical density
PSMF	-	Phenylmthylsulfonylfluoride
OFAT	-	One factor at time method
RSM	-	Response surface methodology
rpm	-	Round per minute
T	-	Temperature
U	-	Unit (enzyme activity)
µmol	-	Micromole
˚C	-	Degree Celsius
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer consist of 2-acetamido-2-deoxy-β-D-glucose through β-(1,4) linkage, a derivative of glucose and it is found abundantly in many places throughout the natural world. It is the main structural of the cell walls of fungi, the exoskeletons of arthropods, the outer shell of crustaceans, insects and etc. (Majeti *et al*., 2000). Chitin has been widely used in many industrial fields such as biochemical, food, medical and various chemical industries. It plays roles as an antimicrobial, anticholesterol and antitumor. Chitin is also used in wastewater treatment, dietary fibers, drug deliver and surgical thread (Boller *et al*., 1998).

Chitin is synthesized by an enormous number of living organisms and it is the most abundant polymer after cellulose. The world marine products are rich with chitin and the two main sources are two marine crustaceans, shrimp and crabs. Every year, global annual recovery of chitin from the processing of marine crustacean waste is estimated to be around 37 300 metric tons (Chang *et al*., 2007). Chitin is a commercial interested material due to its excellent properties such as biocompatibility, biodegradability, non-toxicity as well as chemical and physical stability. Today, several countries in Asia, the Caribbean and Scandinavia, produce chitin of crustacean origin on a commercial scale (Mir *et al*., 2008).

Chitinases are digestive enzymes that hydrolyzed β-1,4-glycoside bond in chitin which exist in many organisms, including bacteria, fungi, insects, even plants and human beings in which chitin are not found. The functions of chitinases in various organisms are diverse. In plants, chitinases are involved in defense mechanism against fungal pathogens and insect attack. However some plants produce chitinases due to creating fungal symbioses. Fungal play role as detritivores and also have potential as arthropod pathogens which chitinases is produced in order to invade their hosts by direct penetration of the host cuticle (Fan *et al*., 2007). In insect and crustaceans, chitinases are required for partial degradation of old cuticle while in human, chitinases can be found in gastric juices and they are involved in catabolic activity (Paoletti *et al*., 2007).

Nowadays, there are many techniques have been developed to produce and enhance the production of chitinases and one of them are by using recombinant DNA technology. In this technique, *Escherichia coli* bacteria is one of the most widely studied organism in recombinant chitinase because it is easy to grow, the well-known genetic and good in expression system (Sorensen *et al*., 2005). Recently, a test have been made which chitinases genes isolated from *Serratia marcescens* was introduced into the endophytic bacterium *Pseudomonas fluorescens* to improve the control of *Rhizoctonia solani* on beans and this test showed very effective result in reducing the damage that cause by pathogen. Besides that, attempts have been made to exploit these antifungal proteins to develop disease- resistant transgenic crop plants. Chitinases genes from rice were recognized have potential to enhance plant resistance against fungal disease in many plants including strawberry, cucumber and tobacco (Yan *et al.*, 2007).

The cloning and expression techniques of the recombinant chitinases genes are done in order to evaluate its antifungal potential against major phytopathogenic fungi (Lee *et al.*, 2006). There are many researches indicate that chitinases purified from plants and bacteria have antifungal activity on pathogens in vitro (Yan *et al.*, 2007). These researches are not only stop at plant and bacteria, but have been expanded to other microorganisms and their enzymatic properties are still investigated until now. Polymerase chain reaction (PRC) is one of the common techniques that be used in

chitinases genes cloning and one research of expression of the recombinant chitinases from *A. caviae* have successful by using this technique (Lin *et al.*, 1997). Today, the development in biotechnology field has given the brightness in production of chitinases enzymes and recombinant technique is the best solution to enhance its production.

1.2 Problem Statement

Chemical fungicides and pesticides are extensively used in current farming practices to protect crops against diseases. However, recently their utilization has been concerned since chemical fungicides and pesticides are highly toxic. They can cause environmental contamination and/or the presence of fungicide/pesticides residues in food products and induce pathogen resistance (Chang *et al*., 2007). Numerous synthetic chemicals may lose their effectiveness as a result of revised safety regulations, concern over no target effects or the development of resistance in pathogen and pest populations.

Biological control using microorganisms offers an alternative environmental friendly strategy for controlling agricultural phytopathogens. Chitinases attract the attention as one of the potential candidate for control of phytopathogenic fungi and insect pests. The interest in chitin degrading enzymes (chitinases) and their application in control of pests and fungal pathogens have advanced significantly, because chitin is a major structural component of exoskeleton of arthropods, insects and fungal cell wall.

However, the production of chitinases enzyme in industries is to be one of the major economic variables, estimated to account for 12% of the total production cost and is presently uneconomic due to the high prices of the commercially available chitinase (Suresh *et al*., 1999). A more efficient and economically viable process is essential to reduce the cost production of chitinases. The utilization of chitinase in various applications has received attention in biotechnology field. Therefore, investigate the

optimization conditions on expression of chitinase should be concerned in order to improve its productivity process, maximum its yield and reducing its production cost.

1.3 Objective of Study

The aim of this study is to optimize the inducers concentration toward the production of recombinant chitinase.

1.4 Scopes of Study

The scopes of this study on the production of recombinant chitinase are:

i. To identify the best concentration of inducers (IPTG and lactose) toward expression of recombinant chitinase in *Escherichia coli* using conventional method.

ii. To optimize inducers concentration (IPTG and lactose) on expression of recombinant chitinase using Response Surface Methodology (RSM).

CHAPTER 2

LITERATURE REVIEW

2.1 Chitin

Chitin is an unbranched polysaccharide composed primarily of β-1, 4 linked N-acetyl D-glucosamine residues with occasional glucosamine residues. The β-1,4-glycoside bond in chitin is hydrolyzed into G1cNAc monomers by interaction between two enzymes, chitinase and β- N- acetyiglucosaminidase (Dahiya *et al*., 2005).

Chitin is easily found in the marine ecosystems because it is the main component of crustacean shells such as crab and shrimp. Every year, more than 10^{11} metric tons of chitin was obtained only in aquatic biosphere. In land, chitin plays a role as the major constituent of the fungi's cell wall and the exoskeleton of many arthropods such insects, beetles, butterflies and etc. Chitin is highly hydrophobic and is insoluble in water and most organic solvent. It is commercial interest due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulose (1.25%) (Majeti *et al*., 2000).

2.2 Chitinases

Chitinases are hydrolytic enzymes that are responsible for the degradation β-1,4-glycoside bond of chitin to its monomer N-acetyl D-glucosamine (Felse and Panda *et al*., 2000). Chitinases are generally found in organisms that either needs to reshape their own chitin or to dissolve and digest the chitin of fungi or animals. Chitinases also play important physiological and ecological roles in chitin metabolism of microorganisms for the production of carbon and energy sources and the antagonism toward target fungi. In higher plants which do not contain chitin constituent, production of chitinase is generally involved in the defense mechanisms against fungal pathogens.

2.2.1 Types of Chitinases

These enzymes have been separated into three types. The first type is endochitinases (EC 3.2.1.14), which break chitin at internal site into chitotetraose, chitotriose and chitobiose. The second type is exochitinase or chitobiosidase, which catalyze the release of chitobiose in a stepwise fashion without the formation of monosaccharide or oligosaccharides. The third type is chitobiases (EC. 3.2.1.30) which split chitobiose, chitotriose and chitotetraose into *N*-acetyl glucosamine (G1cNac) monomers in an exotype fashion (Rohaida, 2007).

2.2.2 Classification of Chitinases

Based on their primary structure and similarities in amino acid sequences, chitinases can be classified into two families of glycosyl hydrolases, 18 and 19. Family18 chitinases break the β-1,4-glycoside bond in G1cNac- G1cNAc and G1cNAc-G1cN, while family 19 chitinases break G1cNac- G1cNAc and G1cN- G1cNAc linkage.

These two families can be separated into six smaller classes which class I, II, IV and V chitinases form the family 19, whereas class III and VI form the family 18. Most of the classes in family 19 are chitinases that only can be found in plants. While the classes in family 18 refer to chitinases for all fungal, animal and bacterial as well as plant chitinases (Iseli *et al*., 1996).

These six classes are identified according to the chitinases characteristics that based on isoelectic pH, signal peptide, location of enzyme, N- terminal sequence, and the inducers. Class I chitinases are endochitinases, whereas Class II chitinases are exochitinases. Class III chitinases do show any similarity to Class I and II chitinases. Although Class IV chitinases show similar characteristics as Class I chitinases, but they are smaller than enzymes Class I. Class V chitinases are nettle lectin precursor that show two chitin binding domain in tandem whereas, Class VI chitinases are all chitinases that no included in Class I, II, III, IV and V chitinases (Rohaida, 2007).

2.2.3 Production of Chitinases

For commercial application, there are three type of fermentations are commonly used in chitinases production namely, liquid batch fermentation, continuous fermentation and fed batch fermentation. Naturally, chitinases are produced by microorganisms and its production is influenced by environment conditions such as temperature, nutrients resources and soil pH. Therefore, all factors that affect chitinases production should be concerned to enhance its production in industrial (Patidar *et al*., 2005).

Media components such as carbon sources, nitrogen sources and agricultural residues are reported to be influenced extracellular chitinases production. Several physical and chemical factors such as aeration, pH, incubation temperature, agitation and inducer also affect chitinases production. For instance, the major role of inducer is

to allow gene expression to occur in order to increase production of protein. In heterologous protein production, high- level of expression protein is important for both research and practical application. Isopropyl β-D-thiogalactopyranoside (IPTG) can be used for high expression of protein. However, IPTG has several considerable drawbacks, including the high cost and its toxicity to humans, which limits its use for production of human therapeutic proteins. Lactose is a potential inducer for engineering products and the using of lactose can enhance the solubility of expression protein. In large-scale production of protein, lactose always been chosen since it is low cost in production and non-toxicity compare to IPTG. However, lactose is needed in large amount for expression purpose since its production of protein is lower than IPTG (Pan *et al*., 2008).

2.2.4 Function of Chitinases

Chitinase is essential for the degradation of chitin, a major component of the fungal cell wall and the exoskeletons of arthropods, such as crustaceans and insects. The functions of chitinase in various organisms are diverse. Chitinase are generally found in organisms that either to reshape their own chitin or to dissolve and digest the chitin of fungi or animals (Chen *et al*., 1997).

In fungal, chitinases and chitin synthase are expressed simultaneously and contribute to cell wall synthesis and wall expansion. In plant, chitinases play role in defense mechanism against infection by phytopathogenic fungi. Naturally, some of bacteria produce chitinases to digest and utilize chitin as a carbon and nitrogen nutrient. Degradation of chitin by bacteria is important in preservative ecosystem and to avoid abundant of chitinous wastes in environment (Boller *et al*., 1998).

2.2.5 Application of Chitinases

There are various applications of chitinases either in industrial or in agricultural and several applications of chitinases were identified including:

2.2.5.1 Pest Control

Insect pathogenic fungi have considerable potential for the biological control of insect pests of plants. The majority of these fungi occur in the Deuteromycotina and Zygomycotina. Many attempts have been made to exploit the Deuteromycotina fungi *Metarhizium anisopliae, Bauveria spp, Nomurac rileyi* and *Verticillium lecaii* for insect control purpose. In this sense, the peritrophic membrane and exoskeleton of insects act as physicochemical barrier to environmental hazard and predators. However, entomopathogenic fungi apparently overcome these kinds of barriers by producing multiple extra cellular enzymes, including chitinolytic and proteolytic enzymes that help to penetrate the cuticle and facilitate infection (Jolles *et al*., 1999). The hydrolytic enzymes used by fungi, insects and other organisms for molting or barrier penetration are potential useful in pest management due to their physiological action is to destroy vital structures such as the exoskeleton or peritrophic membrane insects.

2.2.5.2 Defense and Transgenes in Plants

Numeros plant chitinase genes or cDNAs have been cloned. In a successful case, transgenic tobacco plants were generated which constitutively expressed a bean endochitinase gene under the control of the cauliflower mosaic virus 35S promoter. The transgenic tobacco plants were less susceptible to infection by *Rhizoctonia solant* and either the disease development was delayed or they were not affected at all.

Evaluation of disease development in hybrids plants, heterozygous for each transgenes and homozygous self progeny, showed that combination of the two trangenes gave substantially greater protection against the fungal pathogen *Cercospors nicotianae* than either gene. These data led to the suggestion that combinatorial expression of antifungal genes could be an effective approach to engineering enhanced crop protection against fungal disease (Jolles *et al*., 1999).

2.2.5.3 Mosquito Control

Mosquitoes are a vector agent that carries disease-causing viruses and parasites from person to person without catching the disease themselves and this makes them potential targets for various pest control agents. One research shows that the nematode *Brugia malayi* utilizes a chitinase to break down a proctective chitinous extracellular sheath and the peritrophic membrane to gain entry into the mosquito host (Jolles *et al*., 1999).

2.3 Expression System

Expressed system is actually a combination of an expression vector which its cloned DNA and the host for the vector provide a context to allow foreign gene function in a host cell produce proteins at a high level. Each expression system has distinct advantages, liabilities and the DNA source or the delivery mechanism for the genetic material (Lin *et al*., 1997). Common expression systems include bacteria (such as *E. coli* and *L. lactis*), yeast, insect cells and mammalian cells.