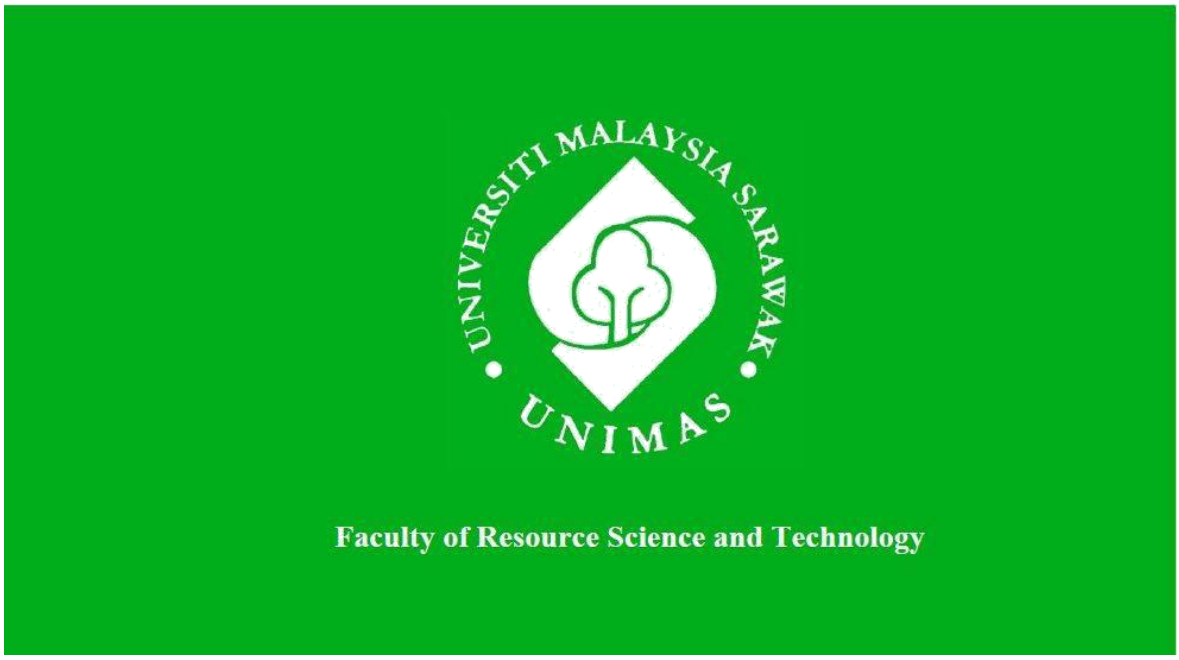

Transformation of Chitinase in Yeast *Pichia Pastoris* GS115

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DECLARATION

I hereby declare that this thesis is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

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LIST OF ABBREVIATIONS

^{o}C	degree Celcius
µL	micro Litre(s)
µg	micro gram(s)
AGE	Agarose Gel Electrophoresis
AOX1	Alcohol Oxidase-1
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
LB	Luria Bertani
LiCl	Lithium Chloride
mL	milli Litre(s)
mM	milli Mole(s)
P. pastoris	*Pichia pastoris*
PEG	polyethylene glycol
rpm	revolutions per minute
SOB	Super Optimal Broth
ss	single-stranded
YPD	Yeast Extract Peptone Dextrose
Zeo^{R}	Zeocin Resistant

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ABSTRACT

Chitinases are enzyme that degrades chitin, the second most abundant polysaccharide after cellulose. Chitin is found in the exoskeleton of many organisms including insects, fungi, yeast and algae. It is also has been found in the internal structures of other vertebrates. Chitin and the chitinolytic enzymes are very useful for their applications in biotechnology sector. Chitinase contribute in human health care especially in human disease like asthma. Chitinases also have a wide-range application in preparation of pharmaceutically important chitooligosaccharides and N-acetylglucosamin. Transformation of chitinase gene in yeast Pichia pastoris GS115 was performed via EasyComp™ transformation. Chitinase gene extracted from *Metroxylan sagu* and has been constructed in pPICZαC was used in this project. The confirmation of insertion in the positive transformant that contain pPICZαC/Chitinase vector was done via colony PCR.

Keywords: Chitinase, chitin, pPICZαC, GS115, *Metroxylan sagu*

ABSTRAK

Kitinase adalah enzim yang mengkatalisis reaksi degradasi kitin, polisakarida kedua terbanyak selepas selulosa. Kitin dijumpai di exoskeleton kebanyakan organisma termasuklah serangga, kulat, yis dan algae. Ia juga didapati di struktur dalaman vertebrata yang lain. Kitin dan kitinase sangat berguna dalam sector bioteknologi. Kitinase sangat penting dalam penjagaan kesihatan manusia terutama asma. Kitinase juga mempunyai pelbagai aplikasi dalam farmaseutikal kitooligosakarida dan N-asetilglukosamin. Transformasi gen kitinase dalam yis *Pichia pastoris* GS115 telah dilakukan menggunakan kaedah EasyComp™. Gen kitinase yang telah diekstrak dari Metroxylan sagu dan dikonstrukkan ke dalam vecktor pPICZαC telah digunakan di dalam projek ini. Pengesahan sisipan di dalam transforman positif yang mengandungi vektor pPICZαC/Chitinase telah dilakukan menggunakan kaedah PCR koloni.

Kata kunci: Kitinase, kitin, pPICZαC, GS115, *Metroxylan sagu*

1.0 INTRODUCTION

Chitinases are one of the glycosyl hydrolases that can catalyze the hydrolytic degradation of chitin, a linear polymer of β-1,4-N-acetylglucosamine (Hamid *et al.*, 2013) with the sizes ranging from 20 kDa to about 90 kDa (Bhattachrya *et al.*, 2007). Chitinases are present in variety of organisms including bacteria, fungi, yeasts, plants, actinomycetes, arthropods and humans. Chitin is found in the outer skeleton of the insects, crabs, shrimps and lobsters, and in the internal structures of other invertebrates (Bhattachrya, *et al.*, 2007). According to Wang and Chang (1997), approximately 75% of the overall weight of shellfish such as crab and shrimp which is disposed as waste, 20-58% of that dry weight is consist of chitin.

The function of chitinase is different in each organism. Plant chitinases involved in plant defense mechanism againt fungal pathogens. In other hand, fungal chitinases were associated with morphogenetic and pathogenesis role (Fan *et al*., 2007). Besides that, the expression of chitinase in every species of plants is also diverse (Yeboah *et al*., 1998). Chitinase also involves in the activation of host defense gene as plants have variety of mechanisms for themselves in order to protect themselves against infection such as pathogen infection (Bishop *et al*., 2000). This activation causes the gene itself to be activated, hence producing a physical and biochemical in the host plant that allowed them to become more resistant to the pathogen attack. According to Xiaohong (2000), the optimum pH for chitinase activity is varies and depends on the types of the species but mostly are in acidic pH.

The chitinolytic enzymes have a wide-range of applications including in pharmaceuticals, biomedical, single-cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, drug deliver, treatment of chitinous waste and enzyme industry (Kumaran *et al*., 2012). There has been a steady increase in the demand of chitin including its derivatives in past several years. This is due to the wide applications of chitinase for various industrial, clinical and pharmaceutical applications. For example, the chitooligomers that are produced by the enzymatic hydrolysis of chitin are useful in human health care (Pandey, 2006).

The problem statement in this study is currently the progress in the industrial production of chitinolytic enzyme is limited and the cost production is high. Due to the high prices of commercially available chitinase, bioprocesses with reduction in the cost of the production should be developed (Pandey, 2006).

The aims of this study are:

- To extract PPICZαC/Chitinase from *Escherichia coli* XL1Blue.
- To transform the chitinase vector into *Pichia pastoris* GS115.
- To confirm the insertion of PPICZαC/Chitinase in *Pichia pastoris* GS115 by colony Polymerase Chain Reaction (PCR).

2.0 LITERATURE REVIEW

2.1 Chitinase

Chitinase is an enzyme that plays an important role to hydrolyze the β-1,4-linkage of chitin. It is known to be produced by a wide range of organisms including bacteria, fungi, higher plants, insects and some vertebrates (Morimoto *et al*., 2001). According to Gohel *et al*. (2006), there are several factors that has been reported to influence the chitinase production by bacteria 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.

Chitinases are able to degrade chitin directly to low molecular weight chitooligomers that are found very useful in industrial, agricultural and medical functions including anti-tumor activity (Yuli *et al*., 2004). Besides that, N-acetylglucosamine (GlcNAc) has the special advantage in the treatment of osteoarthritis (Shiro *et al*., 1996). Due to their role in biocontrol of fungal phytopathogens (Mathivanan *et al*., 1998) and insects (Mendonsa *et al*., 1996), and play the key role in mosquito control, the attention to the chitinases have been increasing.

The pathogens, mainly the protozoan and metazoan used chitin and chitinase, thus causing animal and human diseases. Some of the pathogens are containing chitin coats. These coats are protecting them against both external and internal environment while others that does not have the chitin coats, they attack the host by using chitinase. Therefore, they exploit the chitin-containing structures of the host in order to have a successful infection from one to another (Shahabudin and Vinetz, 1999). Some bacteria have the ability to

produce chitinases; *Streptomyces* (Blaak and Schrempf, 1995), *Alteromonas* (Tsujibo *et al*., 1993), *Escherichia* (West and Colwell, 1984), *Aeromonas* (Sitrit *et al*., 1995).

2.1.1 Types of Chitinase

Chitinase are divided into two major types which are endochitinase and exochitinase (Gohel *et al*., 2006). Most of the plant chitinases are endochitinase including monocotyledons and dicotyledons. The difference between endochitinase and exochitinase is for endochitinase, the chitin is releasing the soluble diacetylchitobiose which is predominates while for exochitinase, the chitin was hydrolyzed by releasing diacetylchitobiose with no mono or oligosaccharide being formed (Xiaohong, 2000).

Endochitinases (E.C 3.2.1.14) split chitin at internal sites randomly thus forming the dimer-diacetylchitobiose and low molecular mass multimers of GlcNAc; chitotriose and chitotetraose (Sahai and Manocha, 1993). Felse and Panda (1999) reported that the activity of exochitinase is defined as the "progressive action starting at the non-reducing ends of chitin with the release of successive diacetylchitobiose unit". Exochitinase are further divided into two subcategories which are chitobiosidases (E.C 3.2.1.29) and 1-4-β-glucosaminidases (E.C 3.2.1.30) (Harman *et al*., 1993). Chitobiosidases are involved in catalyzing the release of di-acetylchitobiose starting from the non-reducing end of the chitin microfibril while 1-4- β-glucosaminidases cleaved the oligomeric products of endochitinases and chitobiosidases and generating monomers of GlcNAc (Sahai and Manocha, 1993). Figure 2.1 shows the three dimensional structure of chitinase.

2.1.2 Classification of Chitinase

Due to the amino acid sequences, five classes of chitinases have been proposed and can be classified under the family 18 and 19 of glycosyl hydrolases (Henrissat and Bairoch, 1993). Chitinase from bacteria, fungi (classes III and V), viruses, animals, mammals and in some plants are categorized into family 18. In family 19, the chitinase are from the plant chitinases of class I, II and IV (Hart *et al*., 1995). β-*N*-acetylhexosaminidase from human, bacteria and *Streptomycetes* are included in family 20 (Dahiya *et al*., 2005).

The sub-classification of the chitinolytic enzyme is also based on other factors; N-terminal sequence, isoelectric pH, localization of the enzyme, signal peptide and inducers. According to Patil *et al*., (2000), the class I chitinases have been found in plants, whereby the class II chitinases are found in plants, fungi and also bacteria. The sequence of class III chitinases is different from the sequence of class I and II. Class I and class IV chitinases have similar characteristics and immunological properties but class IV chitinases are significantly smaller than class I chitinases.

The chitinases of the different families do not share the amino acid sequence similarity and they are likely to evolve from different ancestors. In family 18, the chitinases consists of a number of conserved repeats of amino acids. They consist of an enzyme core that has 8 strands of parallel β-sheets and forming a barrel laid down the α-helices which forms a ring towards the outside (Gooday, 1999). The glycosyl hydrolase family 18 chitinases has the capability to catalyze the transglycosylation reactions (Andres *et al*., 2012).

2.1.3 Uses of Chitinase

Chitinase have various function either for the organism itself or for industrial sectors. In bacteria, chitinase plays the important role in nutrition and parasitism while in fungi, protozoa and invertebrates, chitinase is important in morphogenesis (Reetarani *et al*., 1999). Apart from that, chitinase are also involved in plant defend mechanism (Dahiya *et al*., 2006). In human, chitinase activity takes part in serum that has recently been described (Reetarani *et al*., 1999). Baculovirus that are very useful for biological control of insect pests also produce chitinase for pathogenesis (Deising *et al*., 1995). One of the possible roles of chitinase is to defense against fungal pathogen. The fungal chitinase are required for hyphal growth (Deising *et al*., 1995).

Moreover, chitinases are useful in converting chitin-containing biomass into advantageous components. It can be exploited for their use in control of fungal and insect pathogens of plants (Melchers and Stuiver, 2000). Besides that, the activity of chitinase also acts as an indicator that showing the activity of fungi in soil. Roberts and Selitrennikoff (1988) reported that, there is a strong association between chitinase activity and fungal population in the soil. The correlation of chitinase activity with the content of fungus-specific indicator molecules also has been reported by Miller et al. (1998).

Chitinases also useful in agriculture field and in mosquito control. It can be exploited as additives in order to supplement to the frequently used in insecticides and fungicides. This is to make them more potent and minimize the concentration of the chemically synthesized active agents which are harmful to health and also to the environment

(Giambattista *et al*., 2001). Besides that, chitinase also helps in bioconversion of chitin waste to fertilizer (Sakai *et al*., 1998)

2.1.4 Medicinal Functions of Chitinases

Chitooligosaccharides have an enormous potential in pharmaceutical. They acts as elicitors of plant defense and also have a potential to be used in human medicines such as anti-tumor activity as shown by chitohexaose and chitoheptaose (Shahabudin and Vinetz, 1999). GlcNAc is an anti-inflammatory drug and it is synthesized from glucose which then incorporated into glycoproteins and glycosaminoglycans. Moreover, chitin and chitin binding proteins can be explored for the recognition of fungal infections in humans (Laine and Lo, 1996).

In human health care, chitinases are also has an important function. Another application of chitinase in medical is it has been recommended in increasing the activity of anti-fungal drugs in therapy for fungal diseases (Orunsi and Trinci, 1985). Therefore, they have been use in anti-fungal creams and lotions. Contact lenses, artificial skin and surgical stitches are the examples of some artificial medical articles that are formed from chitin derivatives. This is due to these derivatives are non-toxic, non-allergic, biocompatible and biodegradable (Muzzarelli, 1997).

Chitinases are also important as an effector of host defense in the mammalian immune system. For instance, human that are deficient in chitotriosidase have an increased rate of microfilarial infection. This is due to the suppressed chitinolytic activity thus allowing the parasite to thrive into the host. Therefore, the recombinant human chitotriosidase shows the inhibiton of *Candida albicans* hyphae formation *in vitro* which reducing the mortality in mouse models (Chang *et al*., 2001).

2.2 *Pichia pastoris*

2.2.1 General Characteristics of *Pichia Pastoris*

P. pastoris is a species of methylotropic yeast and has been wide used in protein expression (Cregg *et al*., 2009). *P. pastoris* has several properties that make it suitable for protein expression; high growth rate, able to grow on a simple and inexpensive medium. *P. pastoris* can grow either in shake flasks or a fermenter that makes it suitable for both small and large scale production. As a eukaryote, *P. pastoris* has many advantages of higher eukaryotic expression systems including protein processing, protein folding, and posttranslational modification.

P. pastoris are also easy to manipulate as *Escherichia coli* and *Saccharomyces cerevisiae*. As a yeast, *P. pastoris* also shares the advantages of molecular and genetic manipulations with *Saccharomyces* but *P. pastoris* has a higher heterologous protein expression levels with the advantage of 10- to 100-fold (Daly & Hearn, 2005). The genotypes of *Pichia* strains that will be used in this project is GS115 that has a mutation in the histidinol dehydrogenase gene (*his4*). GS115 will grow on complex medium such as YPD and on minimal media with the aid of histidine (Invitrogen, 2010).

2.2.2 Alcohol Oxidase Proteins (*AOX*1 and *AOX*2)

There are two alcohol oxidase gene in *P. pastoris* which are *AOX1* and *AOX2*. *AOX1* and *AOX2* allow *P. pastoris* to use methanol as a carbon and also energy source. The gene for the desired protein is usually introduced under the control of the *AOX1* promoter,

which means that protein production can be induced by the addition of methanol (Cregg *et al*.,2009).

The expression of *AOX*1 gene induced by methanol to a very high level, usually ≥30% of total soluble protein in cells grown with methanol. *AOX*2 is about 97% homologous to *AOX*1 but the growth on methanol is much slower than with *AOX*1. This slow growth on methanol allows the isolation of Mut^S strains (Cregg *et al*.,2009).

2.2.3 pPICZαC

According to Invitrogen (2010), the expression vector system that is being used in *Pichia* host system is the pPICZ vectors. The pPICZ contain the zeocin antibiotic marker, c-myc epitope and a polyhistidine on its C terminal peptide for rapid detection and purification of recombinant protein. pPICZαC is the 3.6 kb vectors used to express and secrete recombinant proteins in *Pichia pastoris*. pPICZαC contain the following elements; *AOX*1 promoter for tightly regulated, methanol induced expression of the gene of interest, α-factor secretion signal for directing secreted expression of the recombinant protein, zeocin resistance gene for selection in *Escherichia coli* and *Pichia*, and C-terminal peptide that contains the c-myc epitope and polyhistidine (6xHis) tag for detection and purification of a recombinant fusion protein. Figure 2.2 in the appendices shows the pPICZαC vector map.

2.3 Zeocin

Zeocin belongs to the bleomycin family of antibiotic. It is a glycopeptide antibiotic and one of the phleomycins from *Streptomyces verticillus*. Zeocin acts as strong anti-bacterial and anti-tumor drugs (Baron *et al*., 1992). According to Baron *et al.* (1992), antibiotics that are classified in this family show a strong toxicity against bacteria, fungi, plants and mammalian cells. Because of the presence of copper ions (Cu^{2+}), zeocin appeared as blue in colour. The copper-chelated form of zeocin is inactive. Once the zeocin enter the cells, the Cu^{2+} is reduced to Cu^{+} and removed. Hence, the zeocin become activated and then can bind to DNA. In order to prevent the inhibition of the drug causing non-selection, the zeocin must be stored at -20°C and thaw on ice before being used. Furthermore, zeocin is sensitive to light and must be stored in dark.

3.0 MATERIALS AND METHODS

3.1 Media Preparation

3.1.1 Preparation of Luria medium

10 g of Tryptone, 5 g of NaCl and5 g of yeast extract were combined and added with deionized water up to 950 ml. For plates, 15 g/L agar powder was added before autoclaving on liquid cycle at 15 psi and 121°C for 20 minutes. After autoclaved, the luria broth was kept at room temperature while the plates were stored at 4°C.

3.1.2 Preparation of Yeast Extract Peptone Dextrose (YEPD) Medium

The three reagents which are 10 g of yeast extract and 20 g of peptone was combined and added with 900 ml deionized water and was sterilized with autoclaving for 20 minutes on liquid cycle. Then, 100 ml of 10X dextrose was added to the solution. For plates, 20 g of agar powder was added prior to autoclaving. The liquid medium was stored at room temperature.

3.1.3 Preparation of Yeast Extract Peptone Dextrose with Sorbitol + Zeocin™ Agar

10 g of yeast extract, 182.2 g of sorbitol and 20 g of peptone was dissolved in 900 ml of deionized water. 20 g of agar powder was added to the solution. Next, it was autoclaved on liquid cycle at 15 psi and 121°C for 20 minutes. 100 ml of 10X dextrose was added. Then, the solution was allowed to cool to approximately 60°C and 1 ml of 100 mg/ml Zeocin™. The solution was poured into the plates and stored at 4°C in dark.

3.1.4 Preparation of Yeast Extract Peptone Dextrose Medium + Zeocin™

10 g of yeast extract and 20 g of peptone were dissolved in 900 ml of deionized water. For plates, 20 g of agar powder was added. The mixture was autoclaved on liquid cycle at 15 psi and 121°C for 20 minutes. Then, the solution was allowed to cool to 60°C and 100 ml of 10X dextrose. Finally, 1.0 ml of 100 mg/ml Zeocin™ was added. The medium was stored at 4°C in dark.