INFLUENCES OF PLASMID FORMS AND ELECTRIC PULSES ON TRANSFORMATION EFFICIENCY IN *PICHIA PASTORIS* USING ELECTROPORATION

RIAZ AHMED

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by Mr. Riaz Ahmed

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Agricultural Biotechnology of Naresuan University

Oral Defense Committee

T. Jongjitvimol ..... Chair

(Associate. Professor. Touchkanin Jongjitvimol, Ph.D.)

Kawee Sujipuli ..... Advisor

(Assistant Professor Kawee Sujipuli, Ph.D.)

Niran Aeksiri ..... Co – Advisor

(Niran Aeksiri, D.V. M., Ph.D.)

Duangporn P. ..... Internal Examiner

(Associate. Professor. Duangporn Premjet, Ph.D.)

..... Internal Examiner

(Pongsanat pongcharoen. Ph.D.)

Approved

.....

(Associate Professor Paisarn Muneesawang, Ph.D.)

Dean of the Graduate School

16 May 2018

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Riaz Ahmed

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Author	Riaz Ahmed
Advisor	Assistant Professor Kawee Sujipuli
Co-Advisor	Niran Aeksiri, Ph.D.
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ABSTRACT

The electroporation systems was widely used for transforming eukaryotic gene. The purpose of this research was to compare effects of plasmid forms and electric pulses on transformation efficiency in yeast using various electroporation approaches. The pPICZαA-*crypt* plasmid, containing the beta-cryptogein gene, was digested and undigested by *Sac*I to produce linearized- and circular-plasmid, respectively. Both plasmid forms were transformed into competent cells of *Pichia pastoris* (Y11430) using either electric pulse of 1.5 or 2.0 KV. The single colony was selected and cultured in YPD medium, containing 100 µg/ml Zeocin™ and successful transformants were confirmed by using PCR-amplification. The result showed that the linearized plasmid in both electric pulses significantly generated higher transformants (average 29.25-30.00 colonies/plate) than the circular plasmid (7.63-8.13 colonies/plate). Twelve putative transformants in each transformation system were assessed successful transformation efficiency through PCR with present of the inserted *crypt* gene and *AOX*I. The result showed that transformation efficiency of the linearized plasmid (12 of 12 transformants) was significantly higher (p=0.01) than that of the circular plasmid (10 of 12 transformants) in both electric pulses. This study indicated that the linearized plasmid might be used for increasing electroporated transformation efficiency in *P. pastoris*. After small scale cultured cells were collected at different times post induction and beta-cryptogein protein expression is analyzed by SDS PAGE in each

time post. Results showed no detection of beta-*crypt* protein expression in all the test samples.

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ABBREVIATIONS

bp	=	base pairs
BMGY	=	buffered glycerol complex-medium
BMMY	=	buffered methanol-complex medium
cpm	=	counts per minute
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleoside triphosphate
EDTA	=	ethylene diamine tetra acetic acid
EtBR	=	ethidium bromide
LB	=	luria-bertani
PCR	=	polymerase chain reaction
rpm	=	revolutions per minute
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
YPD	=	yeast extract peptone dextrose
KDa	=	Kilo Daltons
OD	=	Optical Density
P. pastoris	=	*Pichia pastoris*
UV	=	Ultra violet light
YNB	=	Yeast nitrogen base
P. cryptogea	=	*Phytophthora cryptogea*
NCBI	=	National Center for Biotechnology Information

CHAPTER I

INTRODUCTION

Introduction

The cryptogein, belonging to the peptide group beta-elicitin, is a protein with low molecular weight (10 kDa, with 98 amino acids) and produced by pseudo-fungi *Phytophthora cryptogea*. The protein is regulated by the beta-cryptogein gene *crypt* which encodes a 297 bp nucleotide sequences (O'Donohue et al., 1995). It has been previously proved to enhance the defense properties in plants such as tobacco (Amelot et al., 2011) and also provides a binding site to the fatty acids and phospholipids in plants (Dobes et al., 2004). Generally, the cryptogein protein was produced by using *in vitro* approach where in the *P. cryptogea* was cultured in a medium supplemented with asparagine and nitrogen, and grown under dark condition at 26°C for 28 days. However, several limitations about this approach was presented to be time-consuming, difficult to grow in culture conditions, and inadequacy of proteins produced (Terce-Laforgue et al., 1992).

Nowadays, recombinant proteins are widely produced by using commercial expression systems in bacteria and yeast due to the simplicity of the system, high amount of produced proteins and the protein purification process is easy to be handled (Weidner et al., 2010). Previous reports have pointed out that genes encoded in eukaryotic proteins were lowly expressed in the bacteria system. For example, the beta-cryptogein protein production in *Escherichia coli (E. coli)* had lower yield of insoluble proteins because the proteins refolded themselves. To cope up with this issue, another alternative protein production system was presented to express in a eukaryotic cell-like *P. pastoris* (O'Donohue et al., 1995). It was highlighted in their report that the expression vector (pTRC99A) carrying *crypt* gene, in *P. pastoris* was able to produce soluble proteins secreted into culture medium. As a result, *P. pastoris* expression systems gives a significant advantage for the production of many heterologous eukaryotic proteins.

Traditionally, beta-cryptogein protein is being mass-produced through the fungal culture technique. The crude protein was directly sprayed or injected into the plant leaves or stems. However, the method is costly, time-consuming and produces a low amount of protein quality and quantity. The expression system of recombinant DNA technique in yeast is very effective and robust. It has been successfully used for many protein productions. However, several limitations was pointed out in the gene transformation process. Transformation systems in the eukaryotic cell, like that in yeast, have two commonly main methods (chemical and electroporation). In the chemical method, the competent cells are prepared by using calcium chloride ($CaCl_2$) whose ions have two main functions for generating temporary pores at the membrane and binding foreign DNA which is able to transfer into the competent cell (Oswald, 2007). However, the disadvantage of the method is that its process requires a high amount of plasmid DNA, it's time-consuming and has low transformation efficiency (DePalma, 2014).

On the other hand, transformation efficiency using the electroporation method relies on electric pulses, which are high potential to create many temporary pores in the cell membrane, resulting in that the foreign DNA is easily transferred into the cell. In the findings of Wu, & Letchworth (2004), it was reported that electric pulse at 1.5 kV revealed higher transformation efficiency in yeast than 2.0 KV. Moreover, plasmid forms (circular and linear) also affect transformation efficiency as reflected in the research works of Calmels et al. (1991) and Ahmad et al. (2014), stating that using linearized plasmid in gene transformation highly increased its efficiency up to 2-3 folds as compared to circular plasmid. In these reports, different genes were put on evaluation and to our knowledge, beta-cryptogein was not being expressed yet in *P. pastoris* after the electroporation method. Hence in this study, the expression of beta-cryptogein using the recombinant DNA technique in *P. pastoris* was investigated, including the efficiency of transformation method through electroporation using different electric pulses and plasmid forms was also evaluated.

Objective of the study

Objective of the study was to compare the different factors affecting the transformation efficiency in yeast competent cell using the electroporation method.

Scope of the study

The beta-cryptogein was synthesised and inserted into pPICZαA vector. The pPICZαA carrying beta-cryptogein was transformed in *P. pastoris* by electroporation. In electroporation process, two different electric pulses (1.5 and 2.0 KV) were carried out in two types of plasmids (linearized and circular) carrying the inserted gene. The successful transformation efficiency was determined by using PCR assay. The *P. pastoris*, containing pPICZαA-*crypt* will be further used to eveluate small expression of beta-cryptogien protein.

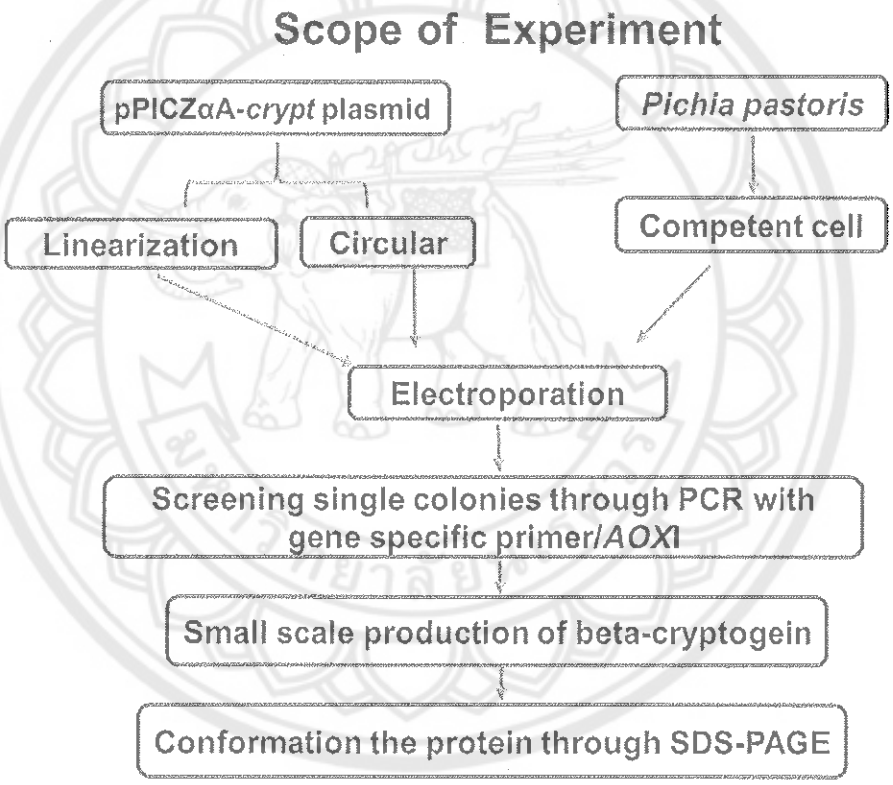

Figure 1 Flow chart of the study

Terminology

1. Beta-cryptogein

The cryptogein, belonging to the peptide group beta-elicitin, is a protein with low molecular weight (10 kDa, with 98 amino acids) and produced by pseudo-fungi *P. cryptogea* (Majumdar et al., 2012).

2. Central dogma

Central dogma (of molecular biology) is defined as biological information flows in one direction, from DNA to DNA replication and DNA is transcribed into mRNA. Then mRNA is translated into proteins. Thus, biological information flows from DNA to RNA to proteins known as central dogma (Thieffry, & Sarkar, 1998).

3. Electroporation

Transformation systems in eukaryotic cell has generally two main methods (using heat shock method and electroporation). Electroporation is a transformation technique where DNA can pass easily in *P. pastoris* by using electric pulse and it is more successful due to certain reasons such as it requires a very low DNA, more efficient technique and it could be operated by living organisms as well (Wang et al., 2011).

4. Gel electrophoresis

Gel electrophoresis is a technique after amplification of DNA, by PCR then used to separate and study the fragment of macromolecules such as protein, DNA and RNA based on their size and charge from mixed population (Lin-Cereghino et al., 2005).

5. *Pichia pastoris*

There are a lot of yeast species one of them is *P. pastoris*, through competent cell of the *P. pastoris* the DNA can pass easily in it and it is able to produce soluble proteins secreted into culture medium. *P. pastoris* expression systems gives a significant advantage for the production of many heterologous eukaryotic proteins (Lin- Cereghino, & Cregg, 2000).

6. Polymerize Chain Reaction

Polymerize Chain Reaction (PCR). It is the synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Metzker, & Caskey, 2009).

7. SDS-PAGE

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to separate protein from mixture and based on their molecular weight. There are two types of gel (1) stacking gel (2) resolving gel (Ouephanit, 2015).

Hypotheses of the study

In transforming beta-cryptogein in *P. pastoris*

1. The linearized plasmid form may be more efficient in transformation efficiency than circular plasmids

2. The use either 1.5 KV or 2.0 KV electric pulse in electroporation method may affect transformation efficiency in *P. pastoris*

3. The expression system of recombinant protein in yeast tool may be successful for beta-cryptogein production.

CHAPTER II

REVIEW OF RELATED LITERATURE

Transformation of a plasmid into yeast

There are a lot of Transformation method by which the DNA is transformed into a cell. Transformation systems in the eukaryotic cell, like that in yeast, have two commonly main methods chemical and electroporation (Figure 2). In the chemical method, the competent cells are prepared by using calcium chloride ($CaCl_2$) whose ions have two main functions for generating temporary pores at the membrane and binding foreign DNA which is able to transfer into the competent cell (Oswald, 2007). However, the disadvantage of the method is that its process requires a high amount of plasmid DNA, it's time-consuming and has low transformation efficiency (DePalma, 2014). On the other hand, Electroporation has been used widely to transfer DNA into yeast, mammalian, and bacteria in culture for the previous 30 years. Transformation efficiency using the electroporation method relies on electric pulses, which have high potential to create many temporary pores in the cell membrane, resulting in that the foreign DNA is easily transferred into the cell Wu & Letchworth (2004), it was reported that electric pulses 1.5 KV and 2.0 KV transformation efficiency in yeast.

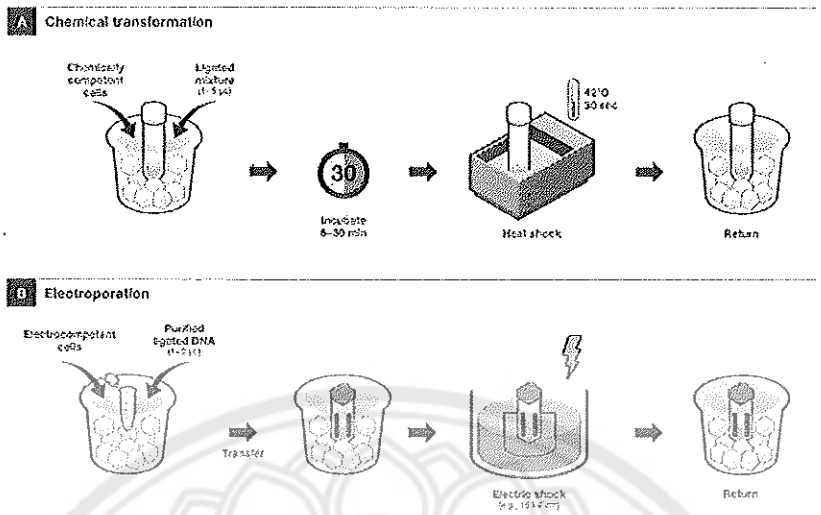

Figure 2 Transformation systems using chemical and electroporation methods

Source: Thermo Fisher Scientific, 2009

Plasmid for the expression system

Plasmids are a small DNA molecule in a cell, which is physically separated from a chromosomal DNA and it can replicate itself freely. Mostly they are in small circular form, double-stranded DNA molecule and they are present in bacteria and in other organisms. There are two most popular form of plasmid forms (circular and linear) which are mostly using for plasmid in an expression system, and these were affected in transformation efficiency. The linearized pPICZαA-*crypt* plasmid was prepared by using *Sac*I and other restriction enzymes according to manufacturer's instruction (Invitrogen, Germany) as reflected in the research works of (Calmels et al., 1991; Ahmad et al., 2014) stating that using a linearized plasmid in gene transformation highly increased its efficiency up to 2-3 folds as compared to the circular plasmid.

pPICZαA Expression system

The pPICZαA vectors were designed for simple cloning and selection because of their ability of high-level expression, and rapid detection and purification of the recombinant protein. Increasing the number of copies of the gene of interest in a *Pichia pastoris* strain can result in higher expression levels (Weidner et al., 2010).

The pPICZαA contains the *AOX*I promoter for tightly regulated and its site allows over-expression of the recombinant gene when placed behind the alcohol oxidase gene promoter (Higgins, & Cregg, 1998). This plasmid confers Zeocin™ resistance as a selective marker and a adds N-terminal alpha-factor signal sequence and C-terminal myc epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant protein inserted into multi cloning site (MCS), many identical copies pPICZαA vector were inserted into yeast genome (Arnau et al., 2006). The pPICZαA vector was used for inducible protein expression in the yeast *P. pastoris*. For smoother downstream production it is important that the proteins produced should be secreted in the culture medium. When proteins are secreted naturally in the extracellular space no modifications are required because it is released naturally by the natural signal. In contrast, those proteins which are produced in the intercellular space require modification. Two most common signals are used for the secretion of proteins in *P. pastoris* expression system. *S. cerevisiae* and alpha mating factor (Macauley-Patrick et al., 2005). This factor is proven to be so fruitful that sometimes it produces more heterologous proteins than the natural system (Cregg et al., 2000). The last few years have been productive, due to designing of *P. pastoris* as host for protein expression. This report refers to well-established tools by *P. pastoris* for high-level of expression vector design, screening commercial applications and engineering host strain of *P. pastoris* derived proteins discussed alongside for numerous protein expression of the membrane (Ahmad et al., 2014).

Beta-cryptogein

The beta-cryptogein which is a sterol scavenging protein produced from the phytopathogenic pseudo-fungus *P. cryptogea* (Ricci et al., 1989; Mikes et al., 1997). The beta-cryptogein gene (with accession number X83001.1) encodes a 297 bp nucleotide sequence as presented in the report of (O'Donohue et al., 1995). This gene translates 98 amino acids which are 10kDa hydrophilic protein containing a hydrophobic pocket with three disulfide bridges (Amelot et al., 2011). Some elicitors such as beta-cryptogein are able to improve the immunes system in tobacco plants (Dokladal et al., 2012). One of the tobacco defence reactions elicitor is a beta-cryptogein protein known as a protein elicitor (Bourque et al., 1999). It is fungal antagonism, the strain of *P. parasitica* (Lascombe et al., 2002; O'Donohue et al. (1996) worked on tobacco plant leaves and infiltrated the protein in it then the protein exhibited defence response and join the necrotic activity.

P. cryptogea is a one of the most resistance opposing tobacco pathogens and exhibited leaf necrosis in tobacco plant (Edreva et al., 2002). Its protein functions are likely a sterol transfer action or a carrier protein using the fluorescent sterol, dihydro ergosterol (Mikes et al., 1997). As shown in Figure 2, this protein has one binding site with strong bond for dihydro ergosterol, additional phospholipidic artificial membranes transfer sterols among protein catalyses, and a molecular activity of beta-cryptogein and for the existence of an extracellular sterol (Amelot et al., 2011).

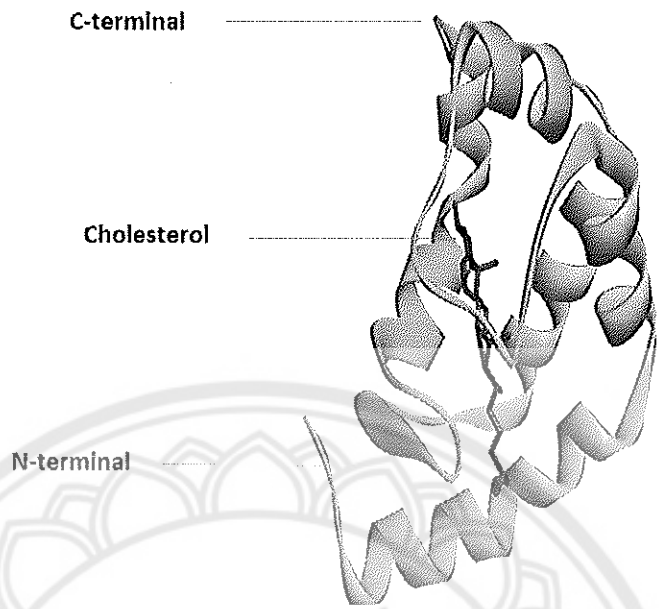

Figure 3 Structure of beta-cryptogein

Source: European Bioinformatics Institute UK, n.d.

Preparation of recombinant plasmid

The sequence of beta-cryptogein was predicted from NCBI gene bank. The nucleotides of beta-cryptogein gene sequence are modified and after confirmation, it was inserted into pPICZ*α*A through cloning with 6 nucleotide sequences recognizing the size of *Eco*RI and *Xba*I added at the 5' and 3' end of the gene, respectively. As the wobble hypothesis that the standard base pairs (between codon of mRNA and anticodon of tRNA) may be rather strict in the first and the second than the third positions of the triplet mRNA codon (Crick, 1966). This was called degenerated codons meaning some tRNA molecules can recognize more than one codon with different nucleotides at the third position of mRNA codon, and it can be encoded the same protein (Alberts et al., 2008). The degenerated codons were preferred for different expression in each organism (Fath et al., 2011). In the present study, the triplet mRNA codons of the original *crypt*-gene (297bp) was modified bases at the third position of each codon by using GeneArt™ and Gene Optimizer™ software to maximize expression of synthetic genes in *P. pastoris* system (developed by Invitrogen, Thermo Fisher Scientific, 2009).

Nucleotide sequences between modified and original sequence of the *crypt*-gene were compared by using the CLUSTAL online program (European Molecular Biology Laboratory (EMBL UK). In this case, this software enabled to optimize and modify codons for maximum gene expression, and it was able to deal with a large number of sequence-related multi parameters involved in different aspects of protein expression in yeast cell such as codon usage, ribosomal entry sites, RNA instability motifs, secondary structure formations (Raab et al., 2010; Fath et al., 2011).

Protein expression in yeast

The increasing demands of plants defense system against pesticides have put scientists and researchers to explore the methods and mechanism to improve plants immune system (Blein, 2002). In yeast, the protein expression is familiar and has a different role in eukaryotic. There are a lot of yeast species and one of them is *P. pastoris* which has the probability to create a high number of recombinant protein in a fast and easy to use expression method (Weidner et al., 2010). Previous research have established tools for *P. pastoris* in high-level of expression vector design, and host strain engineering. Screening commercial applications of *P. pastoris* derived proteins have been used alongside for numerous protein expression of the membrane (Ahmad et al., 2014). Yeast has the capability to produce higher protein production in heterologous expression system (Lin-Cereghino, & Cregg, 2000). The heterologous proteins produced by the *P. pastoris* show homology between the artificial and genomic DNA. It removes the issue of plasmid stability and increases the genetic stability of the desired heterologous genes (Romanos, 1995). At the early time for the production of heterologous proteins, *E. coli* was used as it has the most suitability and also the genome of this bacteria species was well characterized. However, at the same time, it also has limitations such that the prokaryotic cell of *E .coli* does not have membrane-bounded organelles which also include those organelles which are responsible for the production of required proteins after the required modifications (O'Donohue et al., 1995). Cregg et al. (1993) defined and gave some suggestions for selecting good production of foreign proteins hosted in methylotrophic yeast *P. pastoris* and has two main advantages over *S. cerevisiae.* The first advantage is that the promoter used for transcribing the foreign protein is extracted from. *P. pastoris* the *AOX*I gene which is used for the

purpose of foreign protein expression (Vries et al., 1997). The second advantage is that *S. cerevisiae* is a strong fermenter but in the case of *P. pastoris,* it is not a strong fermenter, so during the yeast fermentation process, ethanol is produced in a large amount which goes up to a high toxic level resulting in lower production of desired proteins (Siegel, & Brierley, 1989).

Many publications have successfully used this technique. For medical and industrial purpose, yeast are broadly used for the production of recombinant proteins (Çelik, & Çalık, 2012). Heterologous protein production using host organism's expression system heterologous proteins are produced. Normally for the production of heterologous proteins the species of fungi, mammalian cells and bacteria are used. At a larger scale, they are produced from the species of vertebrates, viruses, fungi, bacteria and plants. There are a lot of yeast expression benefits also tight regulated and efficient promoter (James et al., 2009). The alcohol oxidase1 (*AOX*I) is a promoter gene of *P. pastoris,* commonly used for the expression of foreign genes (Higgins, & Cregg, 1998). Moreover, this is rapid growth on inexpensive minimal medium and its ability to secrete the target protein which simplifies purification (Celik, & Calık, 2012).

CHAPTER III

MATERIALS AND METHODS

All research works and experiments were carried out in the Agriculture Biotechnology laboratory, Faculty of Agriculture, Natural resources and Environment, Naresuan University, Phitsanulok, Thailand.

Chemicals and Research instruments

Among the instruments used in performing all experiments in this study include incubator, autoclave, water bath, vortex, shaker machine, electroporation, micro pulser cuvettes (0.2 cm) and PCR machine and reaction tubes, gel documentation system (gel tank, casting tray, UV trans-illuminator and viewing monitor), sterile glasswares, pasteur pipettes, micro and big, centrifugation machine, microplate reader, Plates, 250 ml flask, baffled flask, autoclaved, 200 ml beaker. PCR reaction tubes, sterile glass and Pasteur pipette.

Chemicals used were yeast extract, peptone powder, dextrose, agar for solid medium, 1 M sorbitol, YPD plates containing Zeocin™, Luria –Bertani broth powder, primers *AOX*I and *crypt*, ampicillin, ethidium bromide, TAE buffer, Acetone, acetic acid, methanol, ethanol and isopropyl alcohol restrication enzyme *Sac*I ans *Xba*I, and Agar, agarose, glycerol, yeast extract, 100 bp DNA ladder, 1 kb DNA ladder, *Xba*I, *Sac*I, PCR Master Mix. Zeocin™ and YNB were purchased from Invitrogen.

Preparation of *E. coli* competent cell

A single colony of *E. coli* strain DH5α was inoculated into 5 ml of LB broth without antibiotic (Pronadisa, Spain). Bacterial cells were cultured at 37°C for 10 hours at 250 rpm. One milliliter of the cell culture was then transferred into 50 ml fresh LB broth and incubated at 37°C for 3 hours at 250 rpm. The Optical Density (OD) was checked at 600nm using a UV visible spectrophotometer (DR04000, Milton Roy Company) and was found to be in the range 0.3-0.4. The culture was then transferred into pre-autoclaved centrifuges tube and placed on ice for 10 minutes and then was

spanned at 4000 rpm for 10 minutes and discarded the supernatant. Pellets were washed with 10 ml (chilled) of 0.1 M calcium chloride was added in the centrifuged mixture and incubated in ice for 10 minutes and was then centrifuged at 4000 rpm at 4°C for 10 minutes and the supernatant was thrown away and only the pallet was left behind. The pellets were then re-suspended using 2 ml of 0.1M $CaCl_2$ and sterile glycerol was added in the mixture of 30 % glycerol and was then incubated for 15 minutes on the ice after mixing it gently. Finally, the solution was transferred in 1.5 ml microcentrifuge 200 µl each and was stored at -80°C until further used.

Beta-cryptogein transformation in *E. coli*

Tubes containing 100 µl of competent cells were thawed on ice. In determining the transformation efficiency, 5 µl of beta-cryptogein was transferred into 100 µl of competent cells tube, was mixed gently and incubated in ice for 15 minutes. After incubation, heat shock was applied to the cells for 45 seconds at 42°C in a water bath without shaking and was then immediately incubated on ice for 2 minutes. One milliliter of LB medium was added to the transformed cell mixture and was spread in different volumes (10 µl, 70 µl, and 100 µl, respectively) on LB media with 100 µg/ml Zeocin™. Finally, the plates were incubated at 37°C for 14 -16 hours.

Selection of positive single colonies and colony PCR

Colony PCR was done taking single colony from the transformants as DNA templates. PCR products were loaded in 1 % agarose gel and run through electrophoresis at 100 V for 40 minutes to detect the positive colonies carrying the inserted beta-cryptogein gene.

Plasmid extraction

A single colony was prepared and cultured (in culture tube) by inoculating 5 ml of Luria- broth medium containing Zeocin™ into the medium at 37°C while shaking at 200 rpm overnight. The next day, the culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14000 x g for 1 minutes. The supernatant was discarded and the pellet was re-suspended in 200 µl of buffer S1 (RNA added). Afterwards, 200 µl of buffer S2 was added to the mixture and mixed thoroughly by

inverting the tube 10 times and left it for some time so that the lysate becomes homologous. After that, 300 µl of C3 buffer was added and mixed immediately by inverting the tube 10 times and then centrifuged at 16000 x g for 10 minutes. The mixture was placed in PM filter column kit and the supernatant obtained from the previous centrifugation was added into the PM column using a pipette and centrifuged again at 14000 x g for 30 seconds. The liquid flow was discarded from the collection tube and was put back to the column. After that, 400 µl of W1 buffer was added to PM column and centrifuged again at 14000 x g for 30 seconds and the flow-through was discarded. W2 buffer (ethanol added) at 600 µl was added into the PM column and centrifuged at 14000 x g for 30 seconds. The supernatant was discarded and the collection tube was placed back to the column. This step was repeated to remove the residual W2 buffer. For DNA elution, the column was placed in a clean 1.5 ml micro-centrifuge tube and 25 µl of buffer E was added into the middle of each PM column and incubated for 5 minute. Centrifugation at 14000 x g for 2 minutes was done after to elute the DNA and the resulting DNA solution from 4 tubes was mixed in one tube. Isopropanol (300 µl) was added to the DNA solution and stored overnight at -20°C. Afterwards, the mixture was centrifuged at 16000 x g for 20 minutes and washed with 400 µl of 70% ethanol. It was placed in the fume hood for 30 minutes afterward to dry the pallet cells completely and then 10 µl of TE was added and mixed using a pipette.

Preparation of competent yeast cells for electroporation

P. pastoris strain was grown on YPD agar medium and incubated at 28°C for 3 days (Figure 3). Its colony was cultured (in culture tube) by inoculating 5 ml of YPD broth into the medium at 30°C while shaking at 200 rpm for overnight. The 1 ml of the cultured *P. pastoris* strain was added into 100 ml fresh YPD-broth medium in a 250 ml conical flask and was grown at 30°C for overnight until the formation of pellet cells. The shaking rate was kept 200 rpm during the culture growth to achieve OD 1.3-1.5 at 600 nm. The culture was centrifuged at a speed of 1500 x g at 4°C for 5 minutes (Invitrogen, 2009).The pellet cells were then re-suspended in 20 ml ice cold sterile water. The pellet cells were centrifuged at 1500 rpm for 5 minutes at 4°C. The pellet was then re-suspended again in 10 ml of cold sorbitol following the same condition as the previous. The same process was repeated using 1 ml of cold 1 M sorbitol. The cell

solution was stored in 1.5 ml microtube on ice for one hour and was used for further processing.

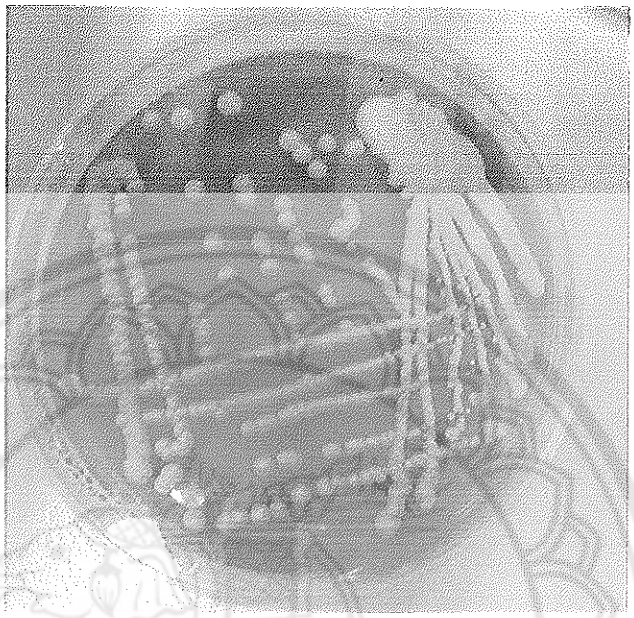

Figure 4 The strain Y11430 of *P. pastoris* used for electroporation on YPD plates

Preparation of pPICZAαA-*crypt* plasmid for transformation

The linearized pPICZαA-*crypt* plasmid was prepared by using *Sac*I Figure 10 according to manufacturer's instruction (Invitrogen, Germany). The reaction mixture contained 7.5 µl of pPICZαA-*crypt* plasmid, 3.0 µl of 10X buffer-L, 1.5 µl of *Sac*I, and 18.0 µl sterilized distilled water making the 30 µl total mixture. The mixture was incubated at 37°C for 16 hours, and at 65°C for 20 minutes for the enzyme activity. Finally, the solution was stored at -20°C until further use (Weidner et al., 2010).

On the other hand, the circular pPICZαA-*crypt* plasmid was prepared as follows: 7.5 µl of circular pPICZαA *crypt* plasmid and 22.5 µl sterilized distilled water following a 30 µl total mixture and was stored at -20°C until further use.

Both circular and linearized plasmids were confirmed on through agarose gel (1%) electrophoresis.

Transformation into *Pichia pastoris* by electroporation screening the yeast cell containing the recombinant vector

The combination between plasmid forms (circular or linear) and electric pulses (1.5 or 2.0 KV) was set up for this experiment to compare transformation efficiency. The experiment was classified into four groups namely linearized plasmid+electric pulse 1.5 KV(M1), circular plasmid+electric pulse 1.5 KV(M2), linearized plasmid+electric pulse 2.0 KV(M3) and circular plasmid+electric pulse 2.0 KV(M4). Yeast competent cells (80 µl), prepared as described above, were mixed with 10 µl of linearized- or circular-plasmid transferred to 0.2 cm micropulser cuvette (Gene Pulser® Cuvette, Bio-Rad, USA) and incubated on ice for 5 minutes. These mixtures were electroporated with two different electric pulses (1.5 or 2.0 KV) by using electroporation (Micropulser™ Bio-Rad, USA). After transformation, cool One milliliter of sorbitol (1 M) was immediately added to the cuvette. The mixture was transferred into a 1.5 ml sterilized microcentrifuge tube and incubated at 30°C without shaking for 2 hours to recover the cells. The transformants (360 µl) were spread on fresh YPD agar plate, containing 100 µg/ml Zeocin™, incubated at 30°C for 3 days until colony formation. Twelve single colonies, resistant to Zeocin™, were selected from individual plate of different transformation methods. Selected colonies were streaked on a fresh master YPD agar plate containing 100 µg/ml Zeocin™ for further confirming successful transformation efficiency. Successful single colonies were selected for PCR and gel electrophoresis was done to confirm the success of the transformation.

Extraction of genomic DNA of yeast

A single colony was picked and cultured (in culture tube) by inoculating it in 2 ml of YPD medium containing Zeocin™ into the medium at 30°C while shaking at 200 rpm for overnight. The next day, the culture was transferred into a 1.5 ml. microcentrifuge tube and centrifugation was done at 5000 rpm for 5 minutes. The supernatant was discarded and 100 µl of liOAc+SDS was added. The mixture was incubated at 70°C for 10 minutes (Looke et al., 2011) afterwards, 300 µl of absolute ethanol was added to the mixture and was mixed by vortexing. Centrifugation at 15000 x g for 3 minutes was done and the supernatant was discarded. The mixture was washed with 300 µl of 70% ethanol by tipping and the liquid was completely removed by

inverting the tube upside down onto a piece of paper for a few seconds and incubated in the fume hood for 30 minutes to dry the pellet cells completely. A total of 100 µl TE was added to the final volume of the mixture, was mixed by vortexing and centrifuged at 15000 x g for 30 seconds.

Screening of single colonies of *Pichia pastoris* by PCR

The single colony after the transformation was confirmed by PCR using specific primers. Primers used and their details were presented in Table 1.

Table 1 Specific primers used in this study

Name of the Gene	Nucleotide size (bp)	Sequence (5'-3')
Beta-cryptogein	309	TCTAGATTACAAGGATGAGCACTTGT
		GAATTCATGGCTTGTACTGCTAC
AOX1 reverse primers:	803	GCAAATGGCATTCTGACATCC
		CGAGTGGTTCCAATTGACAAGC

The single colonies were extracted through the LiOAc DNA extraction method. The inserted gene of interest was confirmed by PCR. For PCR reactions, the One PCR mix protocol (Gene Direx, USA) was used following the set conditions for 35 cycles in (Table 2). The PCR reaction contained 10 µl RNase-free water, 11 µl one PCR, 1 µl forward and reverse primer, and 2 µl of template DNA making 25 µl total reaction. PCR products were run on 1 % agarose gel stained with EtBr through electrophoresis and DNA bands were observed in UV gel doc system.

Table 2 PCR steps used in the modification of 5' and 3' ends of beta-cryptogein

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	59	30 seconds	
Elongation	72	1 minute	
Final Elongation	72	5 minutes	1

Small-scale production of beta-cryptogein

A single colony of transformants (as done and presented in Figure 10) was inoculated in 5 ml BMGY broth medium (1 M potassium phosphate buffer, pH 6.0, 10X YNB stock, 500 X B) containing 100 µg/ml Zeocin™ in a glass tube. Cell culture was grown at 30°C in a shaking at 200 rpm for 24 hours in an incubator (BIOER TECHNOLOGY Model SI-23MC). The cell pellets from 1 ml culture were harvested by centrifuging the mixture at 5000 rpm for 5 minutes at room temperature. The pellets were resuspended in 1 ml sterile water and centrifuged at 5000 rpm for 5 minutes at room temperature. The cell pellets were collected and re-suspended with 5 ml BMGY with 100 µg/ml Zeocin™ and incubated in 30°C with shaking at 200 rpm for 7 days. Methanol was added every 24 hours into the BMGY medium for the next 7 days to maintain the concentration of 0.5%. At certain time after the start of the expression, 1 ml of the culture will be transferred to a 1.5 ml microcentrifuge tube, which will be centrifuged at 1300 x g for 2.5 minutes at room temperature. Supernatant and cell pellets will be transferred to a separate 1.5 ml microcentrifuge tube and stored at -20°C for further use. The samples from different time points (24, 48, 72, 96, 120, 144 and 168 hours) were analyzed to establish the optimal time period for protein expression after induction. At differently expressed proteins, the quality and quantity of target protein as the beta-cryptogein are determined by SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The casting frames were set (by clamping two glass plates in the casting frames) on the casting stands. The separating gel solution (12% and 5 %, respectively) was prepared as described in Appendix page. Ten milliliters of the 12% separating gel solution was filled into the gap between the glass plates first using a pipette. After the gel became hard, the 5% stacking gel solution was filled next into the gap between the glass casting frames until it became hard. The well-forming comb was inserted without trapping air under the teeth. The gel was completely polymerized within 30-40 minutes. After the complete gelation of the stacking gel, the comb was taken out. The casting frame was taken out and was set in the cell buffer dam. The running buffer was poured (electrophoresis buffer) into the inner chamber continuously until it reached the surface level required in the outer chamber. The samples were mixed loading buffer and were heated in boiling water for 5-10 minutes. Prepared samples were loaded into the wells without any overflow and the protein marker were loaded in the first lane. After covering the top, anodes was connected. The electrophoresis was established at 120 volts for 80 minutes and the SDS-PAGE run was stopped when the protein marker almost reached the bottom line of the glass plate. And the gel was putted blue staining for overnight on the platform rocker STR6 and then destained for overnight and changed the solution in water till the required band appeared on gel.

Statistical analysis

The data were analyzed by using the Analysis of Variance (ANOVA) method, and mean comparison among treatments (electroporated transformation methods) were calculated by Tukey HSD test at a p-value less than 0.01 as statistical significance using SPSS statistics 17.0 software. All values were expressed as the mean ± standard error (SE).

CHAPTER IV

RESULTS AND DISCUSSION

Preparation of the beta-cryptogein gene

1. Original gene sequence

The beta-cryptogein which is a sterol scavenging protein produced from the phytopathogenic pseudo-fungus *P. cryptogea* (Boissy et al., 1996; Ricci et al., 1989 ; Mikes et al., 1997) The gene (accession number X83001.1) encodes a 297 bp nucleotide sequence (Figure 4) as presented in the report of (O'Donohue., 1995). This gene encoded codon that was able to translate 98 amino acids (Figure 5) with molecular weight 10 kDa and formed to protein containing a hydrophobic pocket with three disulfide bridges (Amelot et al., 2011)

>X83001.1 Artificial sequence gene of beta-cryptogein
5'ATGGTAGCGGCCGCAACCATGGCTTGCACTGCTACTCAGCAGACTGCTGC
TTACAAGACTTTGGTTTCTATCTTGTCTGACGCGTCTTTCAACCAGTGCTCT
ACTGACTCTGGTTACTCTATGTTGACTGCCAAGGCCTTGCCAACTACTGCT
CAGTACAAGTTGATGTGCGCTTCTACTGCATGCAACACTATGATCAAGAAG
ATCGTTATTTGAACCCACCAAACTGCGACTTGACCGTTCCAACTTCCGGAT
TGGTTTTGAACGTTTACTCTTACGCTAACGGATTCTCTAACAAGTGCTCTTC
CTTGTAGCTAGATAGCTGCAGGTGTAG3'

Figure 5 The 297 nucleotide sequence of the beta-cryptogein gene (X83001.1)

Note: Sequence was taken from NCBI (O'Donohue et al., 1995). The start codon was highlighted in green, and stop codon in red.

MACTATQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKALPTTAQYKLMCA
STACNTMIKKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL

Figure 6 The 98 amino acid sequences of the beta-cryptogein gene as taken from NCBI (O'Donohue et al., 1995)

2. Modified sequence of the pPICZαA carrying beta-cryptogein

The sequence of beta-cryptogein was predicted from NCBI gene bank. The nucleotides of beta-cryptogein gene sequence are modified and after confirmation, it was inserted into pPICZαA (Figure 6) through cloning with 6 nucleotide sequences recognizing the size of *Eco*RI and *Xba*I added at the 5' and 3' end of the gene, respectively.

5'GAATTCATGGCTTGTACTGCTACTCAGCAGACTGCTGCTTACAAGACCTT
GGTTTCTATCTTGTCCGACGCCTCCTTCAACCAGTGTTCTACTGATTCTGGT
TACTCCATGTTGACCGCTAAGGCTTTGCCAACTACTGCCCAGTACAAGTTG
ATGTGTGCTTCCACTGCTTGCAACACCATGATCAAGAAGATCGTCACTCTG
AACCCACCAAACTGCGACTTGACTGTTCCAACTTCCGGTTTGGTCTTGAAC
GTTTACTCCTACGCTAACGGTTTCTCCAACAAGTGCTCATCCTTGTAATCTA
GA3'

Figure 7 Modified sequence of the beta-cryptogein gene from NCBI (O'Donohue et al., 1995) with 6 nucleotide of *Eco*RI and *Xba*I which were highlighted in red, added at the 5' and 3' end of the gene, respectively

As the wobble hypothesis that the standard base pairs (between codon of mRNA and anticodon of tRNA) may be rather strict in the first and the second than the third positions of the triplet mRNA codon (Crick, & Francis, 1966). This was called degenerated codons meaning some tRNA molecules can recognize more than one codon with different nucleotides at the third position of mRNA codon, and it can be encoded the same protein (Alberts et al., 2008). The degenerated codons were preferred for different expression in each organism (Fath et al., 2011).

In present study, the triplet mRNA codons of the original *crypt*-gene (297bp) was modified bases at the third position of each codon by using GeneArt™ and Gene Optimizer™ software to maximize expression of synthetic genes in *P. pastoris* system (developed by Invitrogen, Thermo Fisher Scientific, 2009). Nucleotide sequences between modified and original sequence of the *crypt*-gene were compared by using the CLUSTAL online program (European Molecular Biology Laboratory (EMBL), UK). As shown in Figure 7, the result revealed that some codons were modified base types, which were base substation at the third position of mRNA codon of the *crypt* gene. In this case, this software enabled to optimize and modify codons for maximum gene expression, and it was able to deal with a large number of sequence-related multi parameters involved in different aspects of protein expression in yeast cell such as codon usage, ribosomal entry sites, RNA instability motifs, secondary structure formations (Higgins, & Cregg, 1998; Fath et al., 2011).

```
CLUSTAL O(1.2.4) multiple sequence alignment

Original        ------ATGGCTTGCACTGCTACTCAGCAGACTGCTGCTTACAAGACTTTGGTTTCTATC      54
Modified        GAATTCATGGCTTGTACTGCTACTCAGCAGACTGCTGCTTACAAGACCTTGGTTTCTATC      60
                ******* ********************************** ************

Original        TTGTCTGACGCGTCTTTCAACCAGTGCTCTACTGACTCTGGTTACTCTATGTTGACTGCC      114
Modified        TTGTCCGACGCCTCCTTCAACCAGTGTTCTACTGATTCTGGTTACTCCATGTTGACCGCT      120
                ***** ***** ** ********** ******** ********** ******** **

Original        AAGGCCTTGCCAACTACTGCTCAGTACAAGTTGATGTGCGCTTCTACTGCATGCAACACT      174
Modified        AAGGCTTTGCCAACTACTGCCCAGTACAAGTTGATGTGTGCTTCCACTGCTTGCAACACC      180
                ***** ************** ****************** ***** ***** ********

Original        ATGATCAAGAAGATCGTTACTTTGAACCCACCAAACTGCGACTTGACCGTTCCAACTTCC      234
Modified        ATGATCAAGAAGATCGTCACTCTGAACCCACCAAACTGCGACTTGACTGTTCCAACTTCC      240
                ****************** *** ************************** ************

Original        GGATTGGTTTTGAACGTTTACTCTTACGCTAACGGATTCTCTAACAAGTGCTCTTCCTTG      294
Modified        GGTTTGGTCTTGAACGTTTACTCCTACGCTAACGGTTTCTCCAACAAGTGCTCATCCTTG      300
                ** ***** ********** ********** ***** ********** ******

Original        TAG------ 297
Modified        TAATCTAGA 309
                **
```

Figure 8 Comparison of original and modified nucleotide sequences of beta-cryptogein using multiple sequence alignment (CLUSTAL)

Note: The sequence reads and were compare the original gene with modified gene sequence by using the CLUSTAL online program (European Molecular Biology Laboratory (EMBL UK). The start (*) indicates nucleotide similarity

Original amino-acid sequences of beta-cryptogein were compared with modified sequences using multiple sequence alignment of (CLUSTAL). The results of the analysis showed that in Figure 8 all amino acid sequences of modified nucleotide within *crypt* gene were 100% homologs original *crypt* gene sequence.

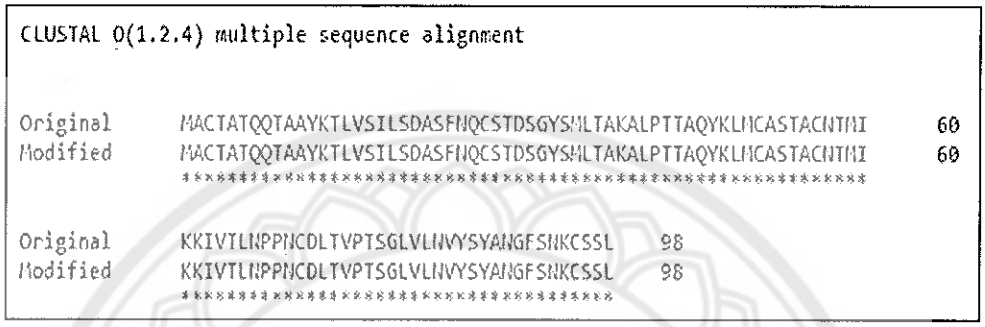

Figure 9 Comparison of 98 original and modified amino acids of beta-cryptogein using multiple sequence alignment (CLUSTAL)

Note: The sequence reads and were compared the original and modified amino acid sequences by using the CLUSTAL online program (European Molecular Biology Laboratory (EMBL UK). The start (*) indicates nucleotide similarity.

Construction of pPICZαA vector

The synthetic gene beta-cryptogein was assembled from synthetic oligonucleotides and the fragment was inserted into pPICZαA (Figure 9). The pPICZαA contains the *AOX*I promoter for tightly regulated and its site allows over-expression of the recombinant gene when placed behind the alcohol oxidase gene promoter with (Higgins, & Cregg, 1998). This plasmid confers Zeocin™ resistance as a selective marker and a c-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant protein inserted into MCS (Arnau et al., 2006). The pPICZαA vector was used for inducible protein expression in the yeast *P. pastoris*.

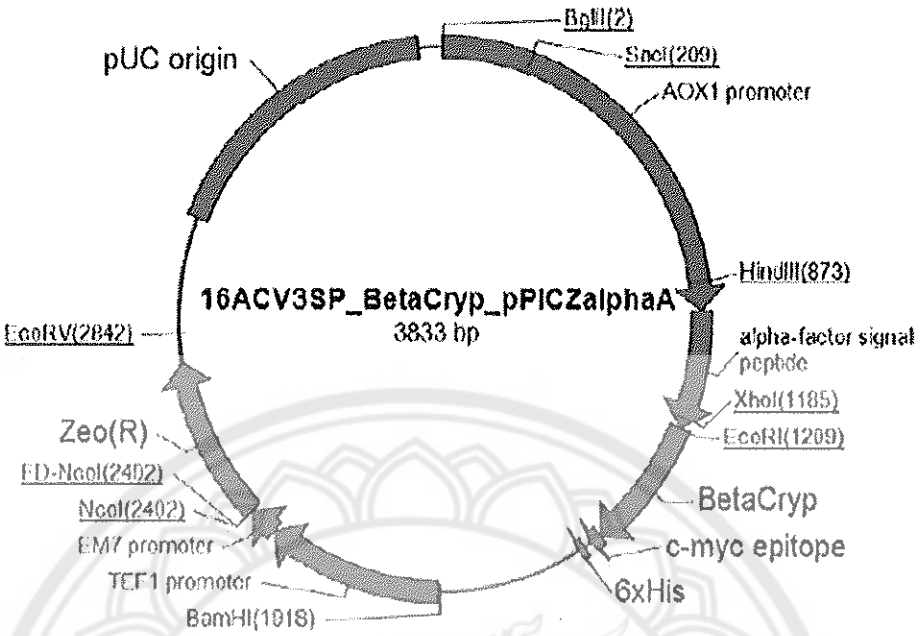

Figure 10 The map of a pPICZαA vector carrying beta-cryptogein gene

Note: A picture of the pPICZαA plasmid showing its important features including, the α-factor signal sequence for extracellular expression, 5'*AOX*I promote methanol induced high level expression of recombinant protein, and the gene that confers Zeocin™ resistance

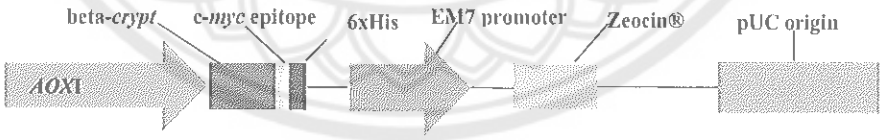

Figure 11 Physical map of the linearized pPICZαA-*crypt* plasmid (3833bp)

Note: *AOX*I promoter region (position at 1-803); beta-cryptogein (position at 941-1270); c-*myc* epitope (position at 1270-1299); 6xHis (position at 1299-1316); EM7 promoter (position at 2074-2241); Zeocin® (position at 2241-2619); and pUC origin (position at 3160-3833)

Evaluation of plasmid forms by gel electrophoresis before transformation

Migration of linearized and circular pPICZαA-*crypt* plasmids in 1% agarose gel after electrophoresis is presented in (Figure 12). Results showed that both plasmid forms migrated differently on the gel indicating that the circular plasmid (corresponding to 3833bp) appeared to be slightly faster than the linearized plasmid indicating that even the same amount of nucleotides was inserted into the same plasmid DNA but in different forms, variation in sizes can be observed. This conforms to the findings of (Ang et al., 2016), stating that the circular pBR322 plasmid was found to have lower molecular weight compared to the linearized pBR322 plasmid after digestion by hydrogen peroxide. The DNA plasmid conformation with the same molecule weight is influenced on its electrophoretic mobility in the agarose gel. Since the circular plasmid form was reduced the size with less space by compacting which cause to be less frictional resistance from the gel, resulting in the migration of this plasmid conformation was able to be faster than other conformations (Cebrian et al., 2015).

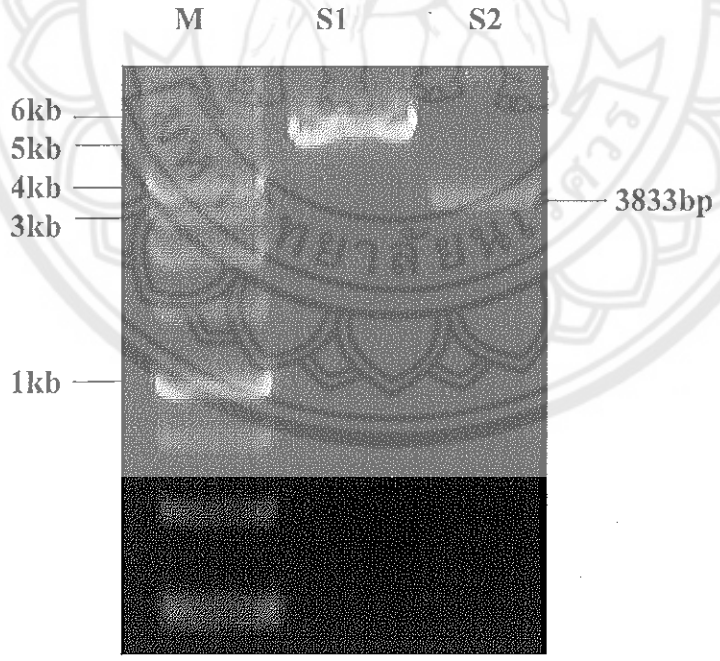

Figure 12 Migration of linearized (S1) and circular (S2) pPICZαA-*crypt* plasmid (corresponding to 3833bp) on 1% agarose gel electrophoresis assay

Note: Lane M represents 1kb DNA RTU Ladder (GeneDireX, USA)

Comparison of transformation methods

Transformation efficiency of four different transformation methods (M) indicating the combination of two plasmid forms (linearized and circular) of pPICZ*α*A-*crypt* and two electric pulses (1.5 and 2.0 KV) is presented in (Figure 13). Results revealed that linearized plasmid digested with *Sac*I gave the higher number of transformed colonies with an average of 29.25 and 30.00 colonies/plate by 1.5 and 2.0 KV, respectively compared to the circular plasmid which yield an average of 8.13 and 7. 63 colonies/plate when treated with 1.5 KV and 2.0 KV, respectively, thus indicating that linearized plasmid is more efficient in transformation than circular plasmid by either of the two electric pulses. Statistical analysis also showed significant differences in the transformation efficiency produced by the two forms of plasmid but showed no significant differences in the treatment of electric pulses at p-value 0.01 (Table 3). These results suggest that linearized plasmid could strongly enhance transformation efficiency in yeast by approximately 3.7 folds compared to the circular plasmid.

These findings conform to previous reports like the one with which revealed that the linearized pUT37 plasmid was found to have highly increased transformation frequency up to 2-3 folds as compared to its associated circular plasmid. Similar findings were found in linearized pDHG25 plasmid digested with *Bam*H1 which generated higher transformed colonies than its circular plasmid (Bessa et al., 2012) also reported that the linearized plasmid p416-GPD digested by *Eco*RI was found to increase the stability of transformation efficiencies in *S. cerevisiae* as compared to its related circular plasmid. Furthermore Schifferdecker et al. (2016), reported that the linearized p892 plasmid with *Hin*dIII yielded higher average number of transformants as compared with the circular p892 plasmid which did not yield any transformants.

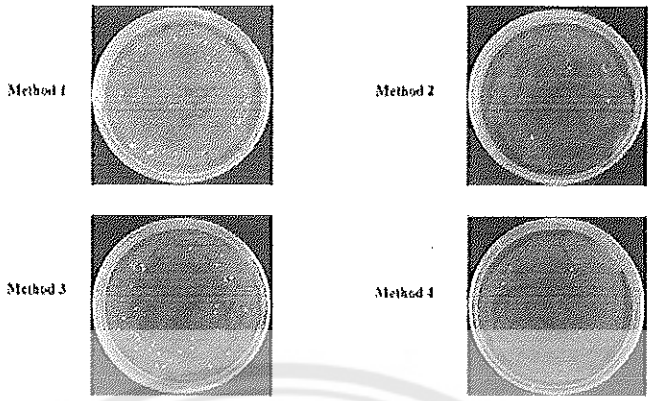

Figure 13 Comparison of successfully transformed colonies of *P. pastoris* among four different electroporated transformation methods

Note: After transformation, transformed yeast cells were cultured on selective YPD medium supplemented with Zeocin™ at 30°C for three days. Each method has been done with eight biological replications, and only one representative per method has been depicted in this Figure

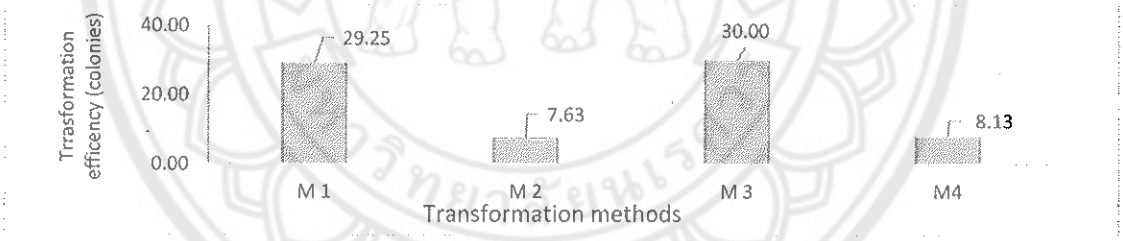

Figure 14 Transformation efficiency of *P. pastoris* in four different electroporated transformation methods

Note: After transformation, the transformed yeast cells were cultured on selective YPD medium supplemented with Zeocin™ at 30°C for three days. Transformation efficiency was determined by the average number of single colonies in the plate. M1, M2, M3, and M4 represent Method 1 (Linearized + 1.5 KV), Method 2 (Circular + 1.5 KV, Method 3 (Linearized + 2.0 KV) and Method 4 (Circular + 2.0 KV), respectively. Data presented are means of 8 replicates. Error bars represent standard error

Table 3 Statiscal analysis on the transformation efficiency of *P. pastoris* in four different electroporated transformation methods

Transformation method	Transformation efficiency
Linearized + 1.5 KV (M1)	29.25 ± 2.11a
Circular + 1.5 KV (M2)	7.63 ± 0.80b
Linearized + 2.0 KV (M3)	30.00 ± 2.05a
Circular + 2.0 KV (M4)	8.13 ± 0.85b

Note: Means with the same letters are not significantly different. Data shown is analyzed using Tukey HSD test relative to P-value < 0.01

Confirmation of successfully transformed colony using PCR

Twelve single-yeast colonies was randomly selected YPD plate from master plates (Figure 15) and was subjected for DNA extraction using the LiOAc DNA extraction method (Looke et al., 2011). DNAs were used as templates for PCR to confirm the success of transformation method. Results showed that all selected yeast transformants from linearized plasmid as treated with either of the two electric pulses positively carried the *crypt*-gene. Meanwhile, only 10 out of the 12 transformants from the circular plasmid either of the two electric pulses carried the inserted *crypt*-gene (Figure 16). In this case, the linearized pPICZαA-*crypt* plasmid with *Sac*I (located at the unique 5'*AOX*I region) have increased transformation efficiency because it might directly transfer into yeast competent cell and its homologous sites (5'*AOX*I region), linked to *Sac*I cleave site, were able to enhance opportunity of synapsis and recombination with the yeast chromosome.

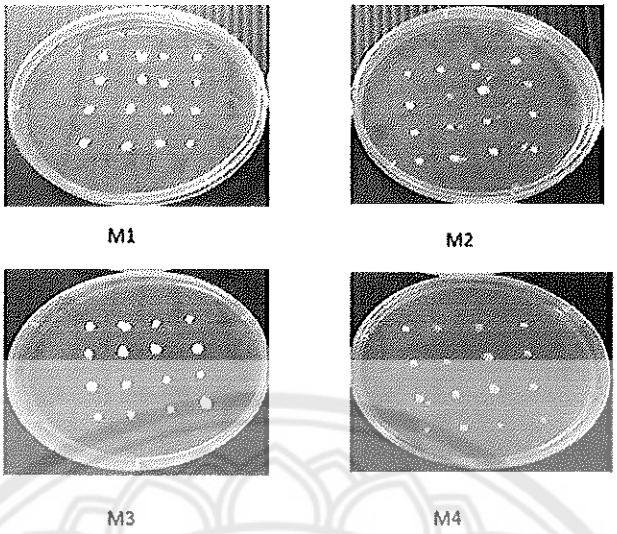

Figure 15 Picture of transformed *P. pastoris* with pPICZαA-*crypt* plasmid picked up on master YPD plates with Zeocin™

Note: M1, M2, M3, and M4 represent Method 1(Linearized + 1.5 KV), Method 2 (Circular + 1.5 KV, Method 3 (Linearized + 2.0 KV) and Method 4 (Circular + 2.0 KV (M3), respectively

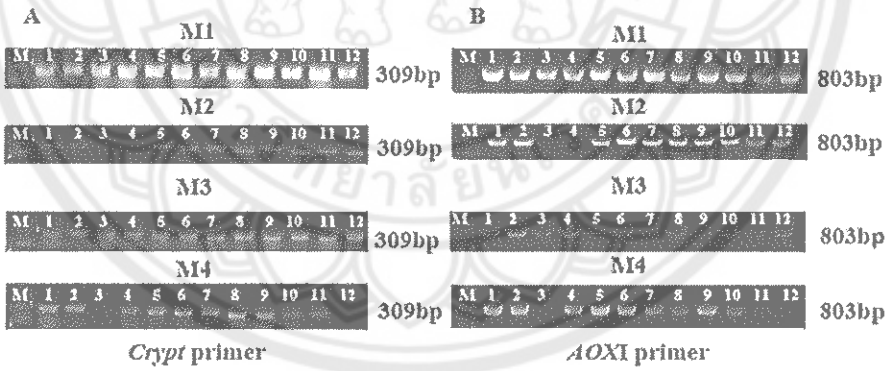

Figure 16 Confirmation of successfully transformed colony using PCR and detected through gel electrophoresis

Note: Migration of PCR products on 1% agarose gel electrophoresis stained with ethidium bromide. DNA templates for PCR amplification were randomly selected from 12 transformed single colonies to confirm the success of gene insertion. Primers used were *crypt* gene specific primer, corresponding to 309 bp (A) and *AOX*I primer corresponding to 803 bp (B)

Small scale of beta- ***crypt*** **in** ***Pichia pastoris***

After successful transformation, single colonies were selected for test inductions of positive beta-*crypt* through PCR. Cultured cells were inoculated in BMGY to generate biomass for induction. The cells from the BMGY culture were then transferred into BMMY media for induction with methanol. Samples were collected after 24 hours and 0.5% methanol was added to the culture every 24 hours for 7 days post induction as it is an established fact that in the presence of methanol media the *AOX*I production increases three to five percent. However, the addition of methanol did not produced any good results it was probably due to unchecked free methanol and oxygen concentration whose levels could only be checked in a fermenter. Cultured cells were collected at different times post induction and beta-cryptogein protein expression is analyzed by SDS PAGE in each time post. Results showed no detection of beta-*crypt* protein expression (corresponding to the size of approximately 10kDa) in all the test samples (Figure 17).

The presence of beta-cryptogein was checked in both supernatant and pellet but was not detected probably due to very low concentration levels or it could also be due to the reason that we don't have a good sophisticated technique for the determining the protein. Moreover, in *P. pastoris* the protein expression is a complex process due to the fact that *P. pastoris* is a eukaryotic organism which makes the protein expression more difficult as compared with *E. coli*. Even for those proteins that are produced at a level of >1g/l in *P. pastoris* the starting shake flask levels are poorly low i.e. <1 mg/l (Fernandez, & Hoeffler, 1998). However, various optimal conditions such as culture temperature, air circulation, methanol-inducer concentration and methanol-feeding strategy for recombinant protein technology should also be considered for further experiments to gain higher expression level of beta-cryptogein protein in *P. pastoris.*

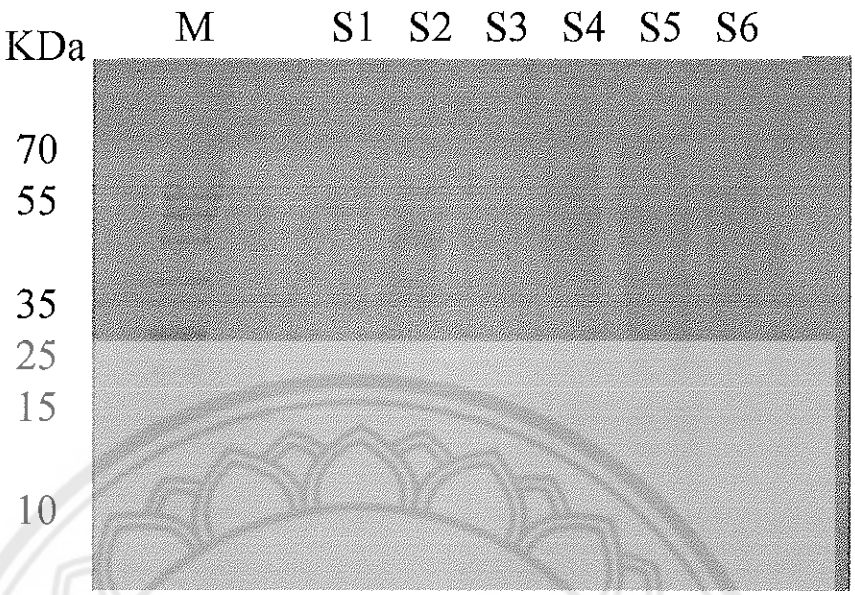

Figure 17 SDS-PAGE analysis of supernatant culture from *P. pastoris* fermentation during the growth time in the absence or presence of beta-cryptogein

Note: Lane M represents Spectra Multicolor Broad Range Protein ladder (Thermo Fisher, Lithuania) and lane S1, S2, S3, S4, S5, and S6 represent the samples of supernatant culture of transformed yeast

CHAPTER V

CONCLUSION

This study was conducted to compare the transformation efficiency in electroporation methods of different electric pulses (1.5 kV and 2.0 KV) combined with plasmid forms (linearized and circular plasmid). The *Pichia pastoris*, carrying pPICZαA-*crypt* was cloned in *E. coli*. The plasmid was extracted and was linearized by *Sac*I. Both linearized and circular plasmid forms were used to transform the beta-cryptogein in *P. pastoris* (strain Y11430) by electroporation with different electric pulses (1.5 kV and 2.0 KV). Average number of single colonies were checked on YPD agar plate's containing100 μg/ml Zeocin™. The result showed that the linearized plasmid in both electric pulses significantly generated higher transformants (average 29.25-30.00 colonies/plate) than the circular plasmid (7.63-8.13 colonies/plate). Twelve putative transformants in each transformation system were assessed successful transformation efficiency through PCR with present of the inserted crypt gene and *AOX*I. The result showed that transformation efficiency of the linearized plasmid (12 of 12 transformants) was significantly higher ($p=0.01$) than the circular plasmid (10 of 12 transformants) in both electric pulses.

Based on the results of this study, the following conclusions were drawn:

1. Linearized plasmid is more efficient in producing successful pPICZαA-*crypt* transormants than circular plasmids.

2. Either electric pulses either 1.5 KV or 2.0 KV can yield good results for transformation efficiency in *P. pastoris*.

3. The small scale of beta-cryptogein protein was examined by SDS PAGE assay, and it showed that both supernatant and pellet of yeast culture could not detected presence of this target protein.

For this result, the further experiments should be recommended that various optimal conditions such as culture temperature, air, methanol-inducer concentration and methanol-feeding strategy for recombinant protein technology should also be considered for further experiments to gain higher expression level of beta-cryptogein protein in *P. pastoris*.

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APPENDIX

APPENDIX STOCK SOLUTIONS AND MEDIA RECIPES

10X YNB (Yeast Nitrogen Base 1 litter)

- 134 grams of yeast nitrogen base (YNB)
- Add 1000ml of distilled water
- Heat solution to dissolve completely
- Filter sterilize the solution and store at 4°C

500X B (0.02% Biotin)

- 20mg biotin
- Add 100ml dH_2O
- Filter sterilize the solution and store at 4°C

10X D (20% Dextrose)

- 200 grams D-glucose
- Add 1000ml dH_2O
- Filter sterilize and store at room temperature

10X M (5 % Methanol)

- 5ml methanol
- Add 95ml dH_2O
- Filter sterilize and store at 4°C

10X GY (10 % Glycerol)

- 100ml glycerol
- Add 900ml dH_2O
- Filter sterilize and store at room temperature

Plain Liquid Luria-Bertani Medium

- 25 grams LB broth powder
- Dissolve in 900ml of dH_2O

- Adjust pH to 7.5
- Make up volume to 1000ml
- Autoclave and store at 4˚C

Luria-Bertani Agar Plate with Antibiotics

- 12.5grams of LB broth powder
- Add 900ml of dH_2O
- Adjust pH to 7.5
- Add 7.5 grams of agar
- Make up volume to 1000ml
- Autoclave for 20minutes and allow to cool to about 55˚C
- Add 0.1 gram Ampicillin and/or 0.034 gram Chloramphenicol
- Pour media into plates and allow to solidify
- Store at 4˚C

Yeast Peptone Dextrose broth medium

- 10 grams yeast extract
- 20 grams peptone
- 20 dextrose
- Add sterile dH_2O to 900ml

Yeast Peptone Dextrose Agar Plate with Zeocin™

- YPD liquid medium
- 10 grams yeast extract
- 20 grams peptone
- 20 grams of dextose
- Add 20 grams of agar
- Autoclave for 20 minutes
- Allow medium to cool to about 55˚C
- Pour medium in plates and allow to solidify
- Store plates containing Zeocin™ in the dark at 4˚C.

Buffered Glycerol-complex Medium

- 10 grams yeast extract
- 20 grams peptone
- Dissolve above in 700 ml distilled water
- Autoclave 20 minutes
- Cool to room temperature and add the following sterile solutions:
- 100ml 1M potassium phosphate buffer, pH 6
- 100ml 10X YNB
- Store media at 4˚C

Buffered Glycerol Methanol Medium

- 10 grams yeast extract
- 20 grams peptone
- Dissolve above in 700ml distilled water
- Autoclave 20 minutes
- Cool to room temperature and add the following sterile solutions:
- 100ml 1M potassium phosphate buffer, pH 6
- 100ml 10X YNB
- 2ml 500X B
- 100ml 10X M
- Store media at 4˚C

Solution for preparing 5% stacking gels for Tris-glycine SDS-PAGE

- H_2O 3.4 ml
- 30% acrylamide mix 0.83 ml
- Tris Cl (1.0M,PH6.8) 0.63
- SDS (10%) 0.05
- Ammonium phosphate (10%) 0.05
- TEMED 0.005

Solution for preparing 12% resolving gels for Tris-glycine SDS-PAGE

- H_2O 2.31 ml
- 30% acrylamide mix 2.8 ml
- Tris Cl (1.5M, PH8.8) 1.75
- SDS (10%) 0.07
- Ammonium phosphate (10%) 0.07
- TEMED 0.003

1 M Sorbitol

- 18.29 grams sorbitol
- Dissolve in 90ml of dH_2O
- Adjust the final volume to 100 ml
- Autoclave and store at 4°C