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Influences of plasmid forms and electric pulses on transformation efficiency in yeast using electroporation

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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 pulseof 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 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*.

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Introduction

The cryptogein,belongs to the peptide beta-elicitin, is a low molecular weight protein (10 kDa) containing 98 amino acids and produced by pseudo-fungi (*Phytophthora cryptogea*). The protein is regulated by the beta-cryptogein (*crypt*; accession number X83001.1) and encoded 297 nucleotide sequences (Gousseau *et al.*, 1995).It has been previously proved to enhance the defensive properties in plants such as tobacco(Amelot *et al.*, 2011).It also provides a binding site to the fatty acids and phospholipids in plants(Dobeš *et al.*, 2004).Because it is a nonspecific carrier of sterol protein which enables the fatty acids and sterols to bind with it. Generally, the cryptogein protein was produced by using the *in vitro* approach. In brief, the *P. cryptogea* was cultured in a medium, supplemented asparagine and nitrogen, and grown under a dark condition at 26˚C for 28 days(Tercé-Laforgue *et al.*,1992). However, the limitation of this approach was time-consuming, laborious, and difficult to grow in culture conditions and less amount of this protein production. Nowadays, recombinant proteins are widely produced by using commercial expression systems in bacteria and yeast. Since this system is simple, able to highly produce proteins and also easy to purify them (Weidner *et al.*, 2010). However, the genes encoded eukaryotic proteins were expressed at the low level in bacterial system. For example, the beta-cryptogein protein production in *Escherichia coli* is low yield with soluble protein. The reason is that protein is refolded itself, and it is an indigenous property. For this limitation, another alternative protein production system was to express in a eukaryoticcell such as*P. pastoris* reported by (O'Donohue *et al.*, 1996). It was first reported that expression vector (pTRC99A), carrying *crypt* gene, was able to produce soluble protein secreted into culture medium in *P. pastoris*. Because of this reason, its expression systems may trend to give a significant advantage for the production of many heterologous eukaryotic proteins. Transformation systems in the eukaryotic cell, such as yeast, have two commonly main methods namely chemical and electroporation methods. For chemical method, the competent cells are mostly 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 this method is that its process requires a high amount of plasmid DNA, time-consuming (Version and Carlsbad, 1999) and low transformation efficiency, compared to electroporation method (Oswald, 2007).

The transformation efficiency using the electroporation method relies on electric pulses, which are high potential to create many temporary pores in the cellmembrane. Resulting that the foreign DNA is easily transferred into the cell. As supported byWu*et al*(2004), reported that the electric pulse at 1.5 KV revealed higher transformation efficiency in yeast than 2.0 KV. Furthermore, the plasmid forms, such as linearized and circular, also played an important role in transformation efficiency. It was supported by Calmels *et al* (1991) and Wang *et al* (2011),reported that linearized plasmid highly increased transformation efficiency as 2-3 folds compared to the circular plasmid.

Therefore, the aim of this study was to compare the transformation efficiency in yeast (*P. pastoris*, strain Y11430) competent cell using different electroporation methods, under the combinations between different plasmid forms and electric pulses.

Materials and methods

Construct of pPICZαA plasmid

The nucleotide sequences of beta-cryptogein (*crypt*) gene (registration number X83001.1 329 bp) from NCBI1, were modified by adding with recognized nucleotide sequences of *Eco*RI (5'-GAATTC-3') and *Xba*I (5'-TCTAGA-3') at the 5' and 3' end, respectively. The *crypt* was inserted into the expression plasmid; pPICZαA (approximately 3.8 kb) (Invitrogen, Germany), and this recombinant plasmid (pPICZαA-*crypt*) was transformed into *E.coli*, strain DH5α using heat shock technique (Lauand Fong, 2008). After that, it was grown on LB agar medium containing 100 µg/ml ampicillin and incubated at

37°C for 14-16 hours. The positive recombinant clones were selected and confirmed by using colony PCR with *crypt* and *AOX*I (alcohol oxidase) primers, which were expected sizes to 329 and 803 bp long respectively. The selected plasmid was extracted by using the kit (Pure Direx plasmid mini PREP kit, Taiwan). The purified plasmid was quantified by using UV-spectrophotometry (Bio Tek®, USA). Its concentration was adjusted to 300 ng/ml with sterilized distilled water, and stored at -20°C until further use in next step.

Preparation of pPICZαA-crypt plasmid for transformation

The linearized pPICZαA-*crypt* plasmid (**Error! Reference source not found.**.) was prepared by using *Sac*I as according to the manufacturer instruction (Invitrogen, Germany). Briefly, the reaction mixture (30 µl) contained circular pPICZαA-*crypt* plasmid 7.5 µl, 10X buffer-L 3.0 µl, *Sac*I 1.5 µl, sterilized distilled water 18.0 µl. The mixture was incubated at 37°C for 16 hours, and the enzyme activity in the mixture was stopped at 65°C for 20 minutes. Finally, the solution was stored at -20°C before further use.

Meanwhile, the circularpPICZαA-*crypt* plasmid (30 µl) was prepared as follows: circular pPICZαA-*crypt* plasmid 7.5 µl and sterilized distilled water 22.5 µl, finally the mixture was stored at -20°C for further use.

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

Preparation of P. pastoris competent cells

The yeast (*P. pastoris*, strain Y11430) competent cell was prepared according to manufacturer instruction of Easy select™ *Pichia* Expression Kit (Invitrogen, Germany). Briefly, the yeast was cultured in 5 ml of YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) and incubated at 30°C with shaking at 200 rpm overnight. The cells were collected by centrifugation at 2000 g for 5 minutes at 4°C. The pellets were dissolved in 1 ml of 1 M cool sorbitol and immediately used for transformation.

Transformation of pPICZαA-crypt plasmids into yeast cells by electroporation

The combination between plasmid forms (circular or linear) and electric pulse (1.5 or 2.0 KV) was set up for this experiment to compare transformation efficiency. The experiment was classified into four groups; linearized plasmid+electric pulse 1.5 KV(named as M1),circular plasmid+electric pulse 1.5 KV(named as M2),linearized plasmid+electric pulse 2.0 KV(named as M3) and circular plasmid+electric pulse 2.0 KV(named as 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 (MicropulserTM Bio-Rad, USA). After transformation, cool sorbitol (1 M) 1 ml 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 YPD agar plate containing 100 µg/ml zeocin™ for further confirming successful transformation efficiency.

Confirmation of successful transformation using PCR

A selected transformants colony was cultured in YPD broth medium, containing zeocin™, and its genomic DNA was extracted using the LiOAc-SDS method(Lõoke *et al*., 2011). The inserted *crypt* gene was confirmed by using PCR amplification with gene-specific primers (*crypt*-reverse 5´-TCTAGATTACAAGGATGAGCACTTGT-3´ and *crypt*-forward 5´-GAATTCATGGCTTGTACTGCTAC-3´) or

plasmid-specific primers (*AOX*I-reverse 5´-GCAAATGGCATTCTGACATCC-3´ and *AOX*I-forward 5´-CGAGTGGTTCCAATTGACAAGC-3´). The PCR condition was performed by an initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of 94˚C for 30 seconds, 59˚C for 30 seconds, and 72˚C for 1 minute, and a final extension at 72˚C for 5 minutes. The PCR product was separated to detect the expected sizes on 1% agarose gel electrophoresis. The expected sizes of PCR product amplified by gene-specific and plasmid-specific primers were corresponded to 329 and 803 bp, respectively.

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).

Results and discussion

Preparation of plasmid for transformation

The pPICZαA plasmid carrying the beta-cryptogein gene (pPICZαA-*crypt*) in *E.coli* strain DH5α was extracted by using the kit (Pure Direxplasmid mini PREP kit, Taiwan).

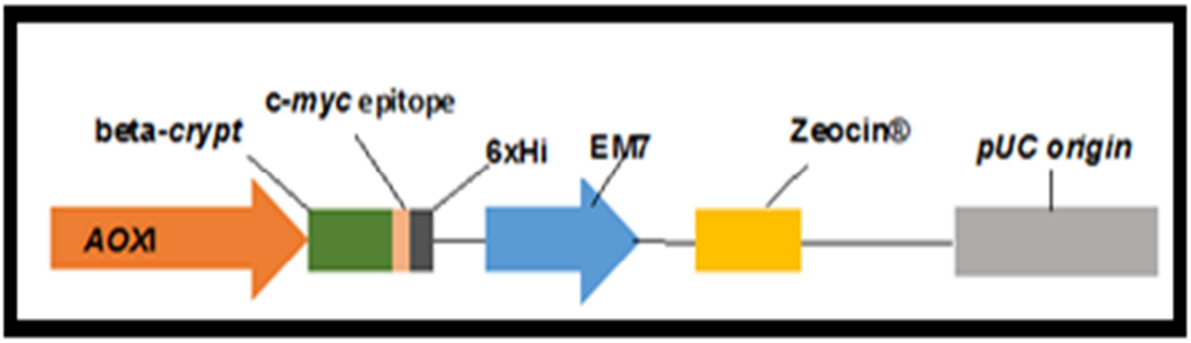


Fig. 1. Physical map of the linearized pPICZαA crypt plasmid (3833bp).

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).

The undigested-circular-plasmid form and the linearized-plasmid form digested by *Sac*I were used for further transformation into *P. pastoris* competent cell, strain Y11430. After treatment with *Sac*I, the undigested or digested-product was determined by 1% agarose gel electrophoresis. As shown in (Fig..) the result found that both pPICZαA-*crypt* plasmid forms were different migrations, which the circular plasmid (corresponded to 3833bp) moved slightly faster than the linearized plasmid on 1% agarose gel electrophoresis. This indicated that the same number of base pairs in plasmid DNA had different migration as previously reported that the circular pBR322 plasmid was expected to lower molecular weight on agarose gel, compared to linearized pBR322 plasmid with digested by hydrogen peroxide (Ping *et al.*, 2016).

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(Cebrián *et al.*, 2015).

Comparison of transformation methods

Four different transformation methods (M) by combining two plasmid forms (linearized and circular) of pPICZαA-*crypt* and two electric pulses(1.5

and 2.0 KV) were compared the transformation efficiency. M1 and M3 represented linearized plasmids of pPICZαA-*crypt* combined with electric pulses of 1.5 and 2.0 KV respectively, and M2 and M4 represented circular plasmids of pPICZαA-*crypt* combined with electric pulses of 1.5 and 2.0 KV respectively.

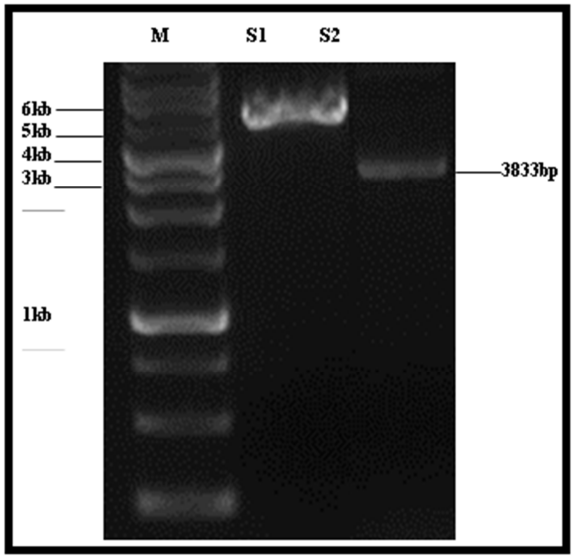


Fig.2. Migration of linearized (S1) and circular (S2) pPICZαA-crypt plasmid (corresponding to 3833bp) on 1% agarose gel electrophoresis assay.
Note: lane M represents 1kb DNA RTU Ladder (GeneDireX, USA).

The result revealed the linearized plasmid (M1 and M3) with *Sac*I gave higher transformed colonies than circular plasmid (M2 and M4) (Fig..). The statistical analysis showed that the linearized plasmid in M1 (29.25 ± 2.11 colonies a plate) and M3 (30.00 ± 2.05 colonies a plate) showed higher average transformation efficiency with highly significant difference at p-value 0.01 than circular plasmid in M2 (8.13 ± 0.85 colonies a plate) and M4 (7.63 ± 0.80 colonies a plate) (**Error! Reference source not found.**). Moreover, the result demonstrated that transformation efficiency in difference electric pulses among 1.5 and 2.0 KV was not significant different at p-value 0.01 (**Error! Reference source not found.**). This indicated that the linearized plasmid could strongly enhance transformation efficiency in yeast competent cells approximately 3.7 folds when comparing to the circular plasmid. This was supported by some previous publications. For example, linearized pUT37 plasmid was highly increased transformation frequency as 2-3 times compared to its circular plasmid (Calmels *et al.*, 1991).The linearized pDHG25 plasmid with *Bam*HI generated higher transformant colonies than its circular plasmid(Sanchez and Aguirre,1996).This also agreed to(Bessa *et al.*,2012), who reported that the linearized plasmid p416-GPD digested by *Eco*RI was shown to significantly increase the stable transformation efficiencies in *Saccharomyces cerevisiae*, compared to its circular plasmid. Futhermore, (Schifferdecker *et al.*, 2016), reported that the linearized p892 plasmid with *Hin*dIII yielded average 68 transformants, but circular p892 plasmid did not give any transformants.

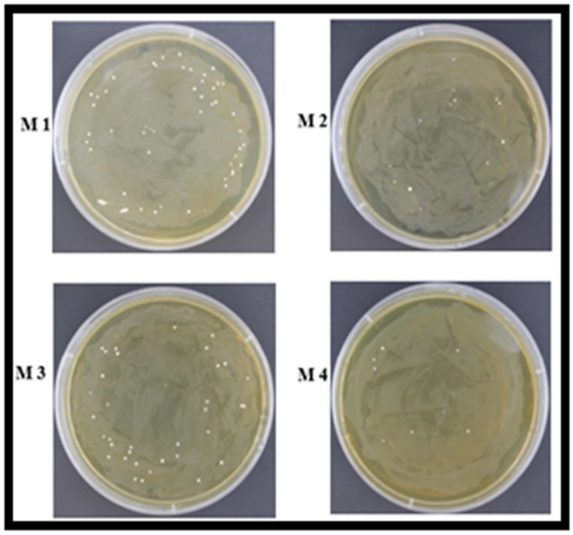


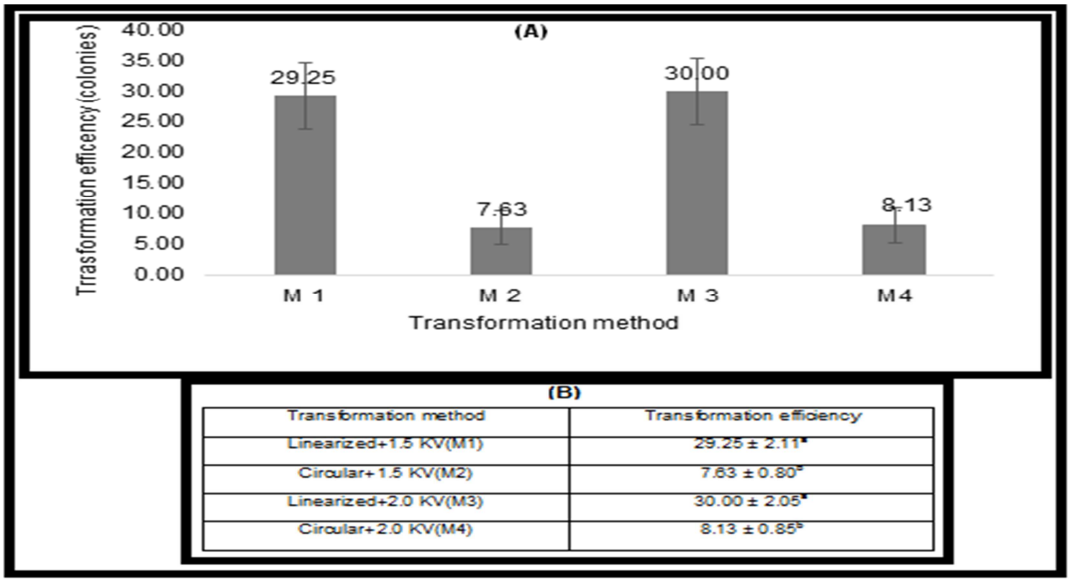
Fig.3. Comparison of successfully transformed colonies of P.pastoris among four different electroporated transformation methods**.**

Note:M1, M2, M3, and M4 represented transformation method of linearized plasmid+electric pulse 1.5 KV, circular plasmid+electric pulse 1.5 KV, linearized plasmid+electric pulse 2.0 KV, and circular plasmid+electric pulse 2.0 KV respectively. After transformation, the 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.

Confirmation of successfully transformed colony using PCR

Twelve putative single-yeast colonies of each transformation method, collected from selective YPD medium plates, were cultured in YPD broth medium supplemented zeocin™ at 30˚C with shaking 200 rpm for 12 hours.



(B)

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

Fig.4. Transformat*ion efficiency of P. pastoris*from 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. The transformation efficiency was calculated by counting a number of single colonies, and bars represented SE (A). Each method has been done with eight biological replications. Values were represented in mean ± SE (B). Different lower case letters (in the same column) corresponded to significant differences at $p < 0.01$, calculated by Tukey HSD test.

The yeast DNA was extracted by LiOAc-SDS method (Lõoke *et al.*, 2011). Its DNA was used to confirm successful transformation using PCR with *crypt*-gene specific primers and *AOX*I primers. The PCR product was separated and visualized by gel electrophoresis. The result found that all selected yeast transformants from M1 and M3 were presented the *crypt*-gene of the insert, which gave the PCR product expected to 329 and 803 bp amplified by *crypt*-gene and *AOX*I primers (

Fig..) respectively.

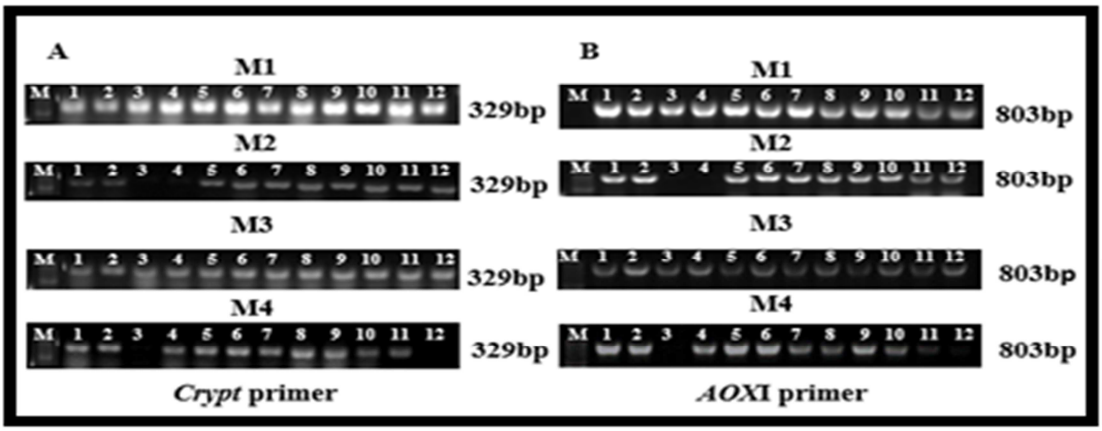


Fig. 5. Confirmation of successfully transformed colony using PCR and visualization by gel electrophoresis.

Note: PCR products were detected target sizes on 1% agarose gel electrophoresis stained with ethidium bromide. DNA templates for PCR amplification was randomly selected from 12 transformed single colonies to confirm the success of gene insertion. Specific primers were used, namely crypt primers corresponding to 329bp and AOXI primer corresponding to 803bp (

Fig..).

From 12 selected yeast transformants, only ten transformants (from M2 and M4) were presented the *crypt*-gene of the insert. In this case, this indicated that the linearized pPICZαA-*crypt* plasmid with *Sac*I (located at the unique 5'*AOX*I region) increased greater transformation efficiency than circular plasmid. The reason of this was that the linearized plasmid might directly transfer into yeast competent cell and it increase long homologous sites were able to enhance opportunity of synapsis and integration with the yeast chromosome (David and Siewers, 2015).

Conclusion

In conclusion, this study demonstrated that the linearized plasmid had significantly higher yeast transformation efficiency than the circular plasmid in both electric pulses (1.5 or 2.0 KV). However, optimal conditions (such as culture temperature, methanol-inducer concentration and methanol-feeding strategy) should also be considered for the further experiment to gain the high expression level of beta-cryptogein protein in *P. pastoris*.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

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