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Construction of Yeast *Pichia pastoris* Expressing the Recombinant p53 as a Secreted Protein into Culture

Tran Thi Thuy Nga[1], Nguyen Quang Hoa[1], Hoang Van Tuyen[1], Do Thi Tuyen[3], Dinh Nho Thai[1,2,*]

[1]*Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam*

[2]*Key Laboratory of Enzyme and Protein Technology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam*

[3]*Enzyme Biotechnology Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*

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Abstract: Human p53 protein has been known as a tumor suppressor and described as "the guardian of the genome", referring to its role in conserving stability of genome by preventing mutation. The recent studies on p53 protein expression have demonstrated the important role and effectiveness of exogenous p53 protein in tumor suppression. In the world, the expression of p53 for therapeutically interest was extensively study but in Vietnam it has not been noticed. In the other hand, *Pichia pastoris* showed a good expression system for many exogenous proteins with a simple cloning work and cheap culture. In this study, codon optimization of gene encoding human p53 protein was performed for suitable expression in *Pichia pastoris* yeast. It was designed as construct of p53 fused with TAT and His-tag sequences (TAT-p53-His). The construct was cloned into pPICZαA expression vector by using *EcoR*I and *Xba*I enzymes to make pPICZαA-TAT-p53-His. Yeast strains containing genes coding for TAT-p53-His was obtained. The integration of TAT-p53-His construct into yeast genome was verified by using PCR with AOX1 primers. The expression of the recombinant TAT-p53-His in the culture of *P. pastoris* X33 was confirmed by SDS-PAGE.

Keywords: Pichia pastoris, gene expression, p53, codon optimization.

1. Introduction

Human protein p53, encoded by gene *TP53,* contains 393 amino acids with mass about 53kDa [1]. Missense point mutations of p53 protein was found in more than 50% of human cancers and most of them are located in its highly conserved protein domains and result in the synthesis of mutant p53 protein lacking DNA-binding activity and failing in tumor-suppressing function [2, 3]. In the normal circumstances, the p53 protein exists with low concentrations because of conditioned ubiquitination by MDM2. Only when the cells are received signal stress or damaged to the

*Corresponding author. Tel.: 84-4-38588579
Email: thaidn@vnu.edu.vn

DNA, p53 is produced with 3 main functions: controlling of the cell cycle in order to prevent the cell not to enter cell division phase; activating the transcription of proteins related to DNA repair and triggering the cell's apoptosis program to prevent the abnormal cells [4, 5].

Pichia pastoris is one of the best systems to the production of heterologous proteins for drug developments [6-8]. There are several advantages of this system, such as, the strong promoter of alcohol oxidase I gene, stably integrate expression vector into genome, a simplified purification procedure for secreted heterologous proteins and post-translational modifications of foreign proteins [9, 10].

Today, the protein therapy has been attractively studied and developed as an important method to treatment of cancer [11]. Recently there are several reports about the additional foreign p53 into culture causing inhibition of the growth and inducing apoptosis of cancer cells [12-14]. With the desire to create recombinant p53 protein for cancer treatment in Vietnam, we conduct this study to express an exogenous p53 in *Pichia pastoris* yeast.

2. Materials and Methods

2.1. Vector, primers and culture strains

E. coli DH5α and X33 *Pichia pastoris* yeast strains were provided by Invitrogen. Vector of pPICZαA was provided by Enzyme Biotechnology Laboratory, IBT. Vector pUC-TAT-p53-His was purchased from IDT company. Primers used for PCR reactions was listed in Table 1.

Table 1. Primers used for PCR reactions

STT	Primers	DNA sequence
1.	EcoRI_Fw	5'-ATGATATCGAATTCTACGGTCG-3'
2.	XbaI_Rv	5'-TACTCGAGTCTAGAAATCAATGATG-3'
3.	AOX1_Fw	3'-GCAAATGGCATTCTGACATCC-5'
4.	AOX1_Rv	5'-GACTGGTTCCAATTGACAAGC-3'

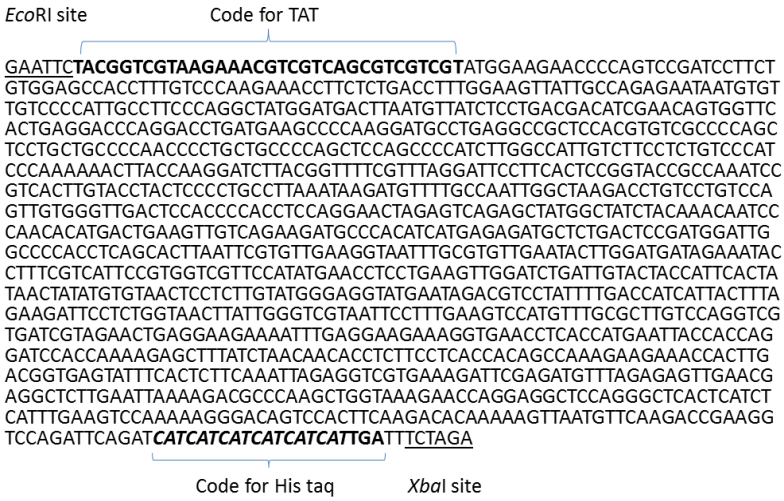

Figure 1. DNA sequence for TAT-p53-His. Recognition sites for restriction enzymes were indicated in underline; sequence coding for TAT was in bold and sequence coding for His-tag was in bold and italic.

2.2. Construction of DNA sequence coding for Tat-p53-His

The mRNA encoding the protein p53 in human from NCBI (AB082923.1) was optimized for suitable expression in *P. pastoris* yeast using GenScript Rare Codon Analysis. 33 nucleotides coding for 11 amino acids of TAT and 18 nucleotides coding for 6X Histidine were fused with optimized p53 to make TAT-p53-His sequence. To facilitate the cloning into expression vector pPICZαA, the recognition sites of two restriction enzymes *EcoR*I and *Xba*I was designed at the ends of TAT-p53-His fragment. The whole structure of TAT-p53-His fragment was artificially synthesized by IDT company and cloned into pUC-TAT-p53-His vector. The codon optimized sequences for TAT-p53-His was presented in Figure 1.

2.3. Construction of PICZαA vector contained TAT-p53-His sequence

pUC-TAT-p53-His vector was transformed into competent *E. coli* DH5α cells by heat-shock and cultured in LB media contained ampicillin. pUC-TAT-p53-His plasmid was extracted by using *GenJET*^{TM} *Plasmid Miniprep Kit* (Fermentas) and TAT-p53-His fragment was cut by couple of restriction enzymes *EcoR*I/*Xba*I and ligated with *T4* DNA ligase with pPICZαA previously digested with the same enzymes (*EcoR*I/*Xba*I) to make recombinant pPICZαA-TAT-p53-His plasmid. This plasmid was transformed into a new competent *E. coli* DH5α strains to select the colonies in LB media contained Zeozin. PCR technique was used to check the clone of TAT-p53 with EcoRI_Fw and XbaI_Rv. Further, TAT-p53-His cloned into pPICZαA was confirmed by DNA sequencing (First Base, Singapore).

2.4. Transformation pPICZαA-TAT-p53-His into yeast and selection of recombinant clones

Pichia pastoris strain (X33) was grown in 100 ml YPD medium (1 % yeast extract, 2 % peptone, 2 % dextrose) at 30^{o}C, 200 rpm until OD_{600} = 1.4-1.6. Cells were collected by centrifugation at 4.000 rpm, 5 min, 4^{o}C. The pellet was dissolved in 100 ml ice-cold water and centrifuged at 4.000 rpm, 5 min, 4^{o}C (repeated twice). The pellet was washed in 4 ml ice-cold 1 M sorbitol and re-suspended in 0.2 ml of 1 M sorbitol and the cells were kept on ice until use. 80 µl above cells were mixed with 5–10 µg of plasmid DNA (pPICZαA-TAT-p53-His vector previously digested with *Sac*I) by pulsed electroporation (1500 V, 25 µF, 200Ω). Immediately after electroporation, 1 ml of ice-cold 1 M sorbitol was added to the cells and incubated for 2 h at 30^{o}C and transformants were selected on YPDS plate contained Zeocin (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol and 100µg/ml Zeocin). Colonies appeared after 3 days incubation were used for colony PCR with AOX1_Fw and AOX1_Rv to investigate the integration of TAT-p53-His construct into yeast genome.

2.5. Expression of p53 in Pichia pastoris X33 yeast

A colony carrying the p53 protein expression vector were cultured foreign protein biosynthesis in BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 4.10^{-5}% biotin, 1% glycerol, pH 6) at 28°C, shaked 250-300 rpm overnight until OD600 about 2-6. Cells were centrifuged and transfered in fresh BMMY (1% yeast extract, 2% peptone, 1.34% YNB, 4.10^{-5}% biotin, 0.5% methanol, pH 6) medium with OD600 approximately equal to 1, and were grown at 28°C, shaked 250-300 rpm. Methanol was added daily to the appropriate concentration of 0.5% to induce p53 recombinant synthesis and was as carbon source for cell growth. Cell density is used to measure the growth of the cells, when OD600 is greater than 3, the samples were diluted 10 times and measured to get accurate results. Cell cultures were collected after 72 hours of incubation and cells were removed by centrifugation at 3.000 rpm for 10 minutes. Extracellular fluids were preserved at -20°C to

evaluate the possibility of p53 synthesis of recombinant strains by SDS-PAGE. The experiments were repeated 3 times and the growth curve is set based on the average value and standard deviation of the measurements.

3. Results and Discussion

3.1. Codon optimization for DNA sequence coding human p53

Using GenScript Rare Codon Analysis tool, we analysed the suitable codon indicators CAI (codon adaptation index) and determined the codons in the sequences of the gene which are present as low frequency usage by *Pichia pastoris* yeast. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. Codons with values lower than 30% are likely to hamper the expression efficiency (Fig. 2A).

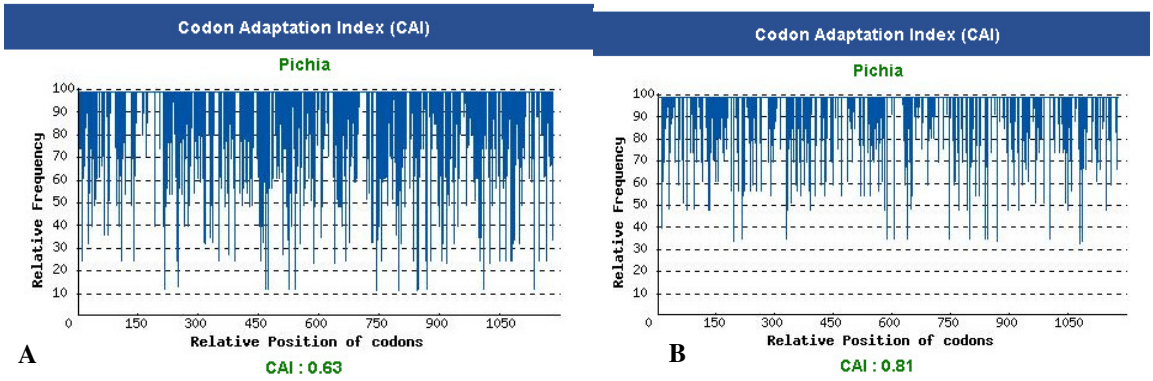

Figure 2. The distribution of codon usage frequency along the length of the p53 coding sequence for expression in *Pichia pastoris*. (A) The p53 coding sequence before codon optimization. (B) The p53 coding sequence after codon optimization.

In this study, the codons having the appropriate index under 30% were changed by the synonymous codons having consistently with 90-100% (Fig. 2B). CAI index analysis results of the p53 coding sequence expression system in *P. pastoris* yeast showed before the codon optimization, sequence of gene had CAI = 0.63, not really suitable for gene expression in yeast. After being replaced by the synonymous codons, CAI index increased 0.81 has improved without codons have a low frequency of use under 30%, instead of the codon has a high frequency of usage (Fig. 2B). Nucleotide sequence coding for TAT, p53, His-tag and restriction enzyme sites were 1247bp, was named shortly as TAT-p53-His. This construct was commercially synthesized and provided as pUC-TAT-p53-His plasmid.

3.2. Construction of the expression vector pPICZαA-TAT-p53-His

TAT-p53-His was cut and collected back from pUC-TAT-p53-His plasmid by pairs of restriction enzymes *EcoR*I/*Xba*I (Fig 3A, lane 2). pPICZαA expression vector also was cut to open the round by this pairs of enzyme (Fig. 3A, lane 4). TAT-p53-His was paired into vector frame of pPICZαA straight circuit by *T4* DNA ligase and trasformed into the cells of *E. coli* DH5α strains. We collected the colony by PCR technique with specific primer pairs for vector (AOX1-Fw/AOX1-Rv). Positive colonies having PCR product of 1.8 kb DNA band on 1% agarose gel (figure 3B, lane 2-9) was selected for extraction plasmids and tested by using restriction enzymes and DNA sequencing.

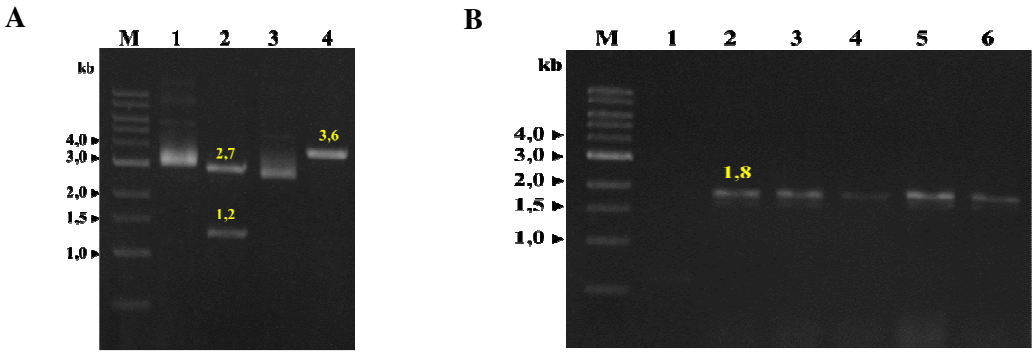

Figure 3. Result of electrophoresis of products of restriction enzyme (A) and PCR reactions (B).
(**A**) Processing the pUC-TAT-p53-His and pPICZαA with *EcoR*I/*Xba*I; Lane 1: intact pUC-TAT-p53-His; Lane 2: pUC-TAT-p53-His plasmid products digested with *EcoR*I/*Xba*I; Lane 3: intact pPICZαA; Lane 4: pPICZαA products digested with *EcoR*I/*Xba*I. Lane M: 1 kb marker.
(**B**) The PCR products of colonies with the pair of primer AOX1-Fw/AOX1-Rv; Lane 1: negative control; Lane 2-9: the PCR products of colonies. Lane M: 1 kb marker.

pPICZαA-TAT-p53-His plasmid was cut by enzyme *Xba*I obtained a DNA band about 4.8 kb (Fig. 4, lane 2); and when dealing with two restriction enzymes *EcoR*I/*Xba*I, producing a DNA fragment of 3.6 kb is the vector pPICZαA and a DNA band about 1.2 kb in size is the length of TAT-p53-His (Fig. 4, lane 3).

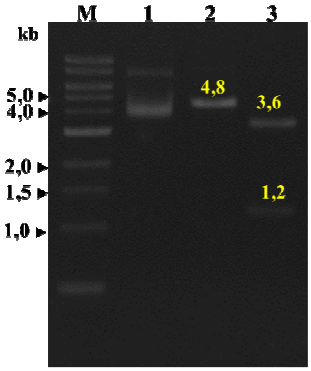

Figure 4. The recombinant vector pPICZαA-TAT-p53-His was extracted from a colony and checked by using restriction enzymes. Lane1: intact pPICZαA-TAT-p53-His vector; Lane 2: product of digesting pPICZαA-TAT-p53-His with *Xba*I; Lane 3: products of digesting pPICZαA-TAT-p53-His with both *EcoR*I/*Xba*I. Lane M: 1 kb marker.

3.3. Construction of P. pastoris X33 strain contained pPICZαA-TAT-p53-His intergrated into genome

To insert TAT-p53-His into the yeast's genome, pPICZαA-TAT-p53-His plasmid was digested with *SacI* and transformed into *Pichia pastoris* X33 strain. According to the protocol manual, pPICZαA-TAT-p53-His plasmid is crossedover and inserted the whole exogenous gene expression structure in AOX1 region in *Pichia* genome. Therefore, AOX1 structural gene in the genome is conserved to produce alcohol oxidase enzyme to convert methanol as a source of carbon for yeast growth. This type of Mut^+ strain of recombinants has been preferably selected because methanol is used as an inducer of foreign protein biosynthesis and is a carbon source for recombinant strains to grow well.

The Mut^+ recombinants obtained and verified by using PCR with AOX1 primers. Results from PCR products electrophoresis of 6 recombinants showed that all of them had two bands: higher band is AOX1 gene in inherent Pichia genome (about 2.2 kb) and lower band is expression structure from recombinant vector (about 1.8 kb) (Fig. 7, lanes 4-9). Therefore, all of the recombinants are Mut^+ strains.

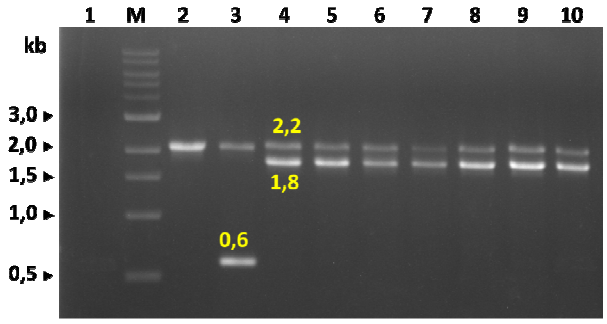

Figure 5. Electrophoresis result of selection of recombinant strains by using PCR with AOX1 primers. Lane 1: negative control; Lane 2: PCR product of *Pichia* genome; Lane 3: PCR product of *Pichia* transformed pPICZαA; Lane 4 to 10: PCR products of pPICαA-TAT-p53-His recombinant strains. Lane M: marker 1 kb.

3.4. Expression of recombinant TAT-p53-His protein

P. pastoris X33 strain carrying plasmids pPICαA-TAT-p53-His and pPICZαA parallel cultured in YP medium with 1% methanol and methanol is added per 24 hours. To track the growth of recombinant strains and the effect of TAT-p53 to the vitality of the transformants, we sampled cultures before adding methanol and measured OD600 culture interval times. After 72 hours of cultivation, the culture mediums were collected and used for SDS-PAGE, Figure 6.

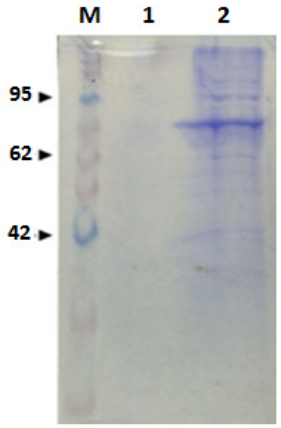

Figure 6. Result of SDS-PAGE with culture media samples. Lane 1: X33 transformed with pPICZαA (control); Lane 2: X33 transformed with pPICZαA-TAT-p53-His; Lane M: protein marker.

In this experiment, TAT-p53-His construct was secreted in the cultures would have 500 amino acids in size (90 amino acids of α factor signal sequence + 11 amino acids of TAT sequence + 393 amino acids of p53 protein + 6x histidine) and about 65-75 kDa. The control was absent of this range indicated that we sucessed in constructing of yeast *P. pastoris* expressing the recombinant TAT-p53-His as a secreted protein into culture. However, there are several extra-cellular proteins in the culture media, the steps of expression optimization and protein purification should be carried out in the future.

4. Conclusion

In this study, codon optimization of gene encoding human p53 protein was performed for suitable expression in *P. pastoris* yeast. It was designed as construct of p53 fused with TAT and His-tag sequences (TAT-p53-His). The construct was cloned into pPICZαA expression vector and transformed into yeast strains. The integration of TAT-p53-His construct into yeast genome was verified and the expression of the recombinant TAT-p53-His in the culture of *P. pastoris* X33 was confirmed by SDS-PAGE. The recombinant yeast strains will be used for further studies in p53 protein expression and its application in cancer therapy.

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Tạo chủng nấm men *Pichia pastoris* nhằm biểu hiện protein tái tổ hợp p53 ra môi trường ngoại bào

Trần Thị Thúy Nga[1], Nguyễn Quang Hòa[1],
Hoàng Văn Tuyến[1], Đỗ Thị Tuyên[3], Đinh Nho Thái[1,2]

[1]*Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam*
[2]*Phòng Thí nghiệm trọng điểm Công nghệ Enzym và Protein, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam*
[3]*Phòng Công nghệ Sinh học Enzyme, Viện Công nghệ Sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam, Số 18 Hoàng Quốc Việt, Cầu Giấy, Hà Nội, Việt Nam*

Tóm tắt: Protein p53 đã được biết đến với vai trò ức chế khối u và điều chỉnh sự phát triển của tế bào trong cơ thể người. Các nghiên cứu gần đây về biểu hiện protein p53 trên thế giới đã chứng minh vai trò quan trọng và hiệu quả của protein p53 ngoại lai trong việc ức chế khối u. Mặc dù biểu hiện protein p53 trên thế giới đã được nghiên cứu nhiều nhưng ở Việt Nam liệu pháp điều trị ung thư sử dụng protein p53 chưa được chú ý. Mặt khác, nấm men *Pichia pastoris* có nhiều lợi thế của hệ thống biểu hiện của sinh vật nhân thực như cải biến protein, cuộn gấp protein, và biến đổi sau dịch mã, trong khi vẫn thao tác dễ dàng như ở *E. coli* hay *S. cerevisiae*. Trong nghiên cứu này, chúng tôi đã thiết kế và tối ưu mã bộ ba của đoạn DNA mang trình tự mã hóa protein p53 ở người và các yếu tố cần thiết cho tinh sạch protein này, gọi là trình tự TAT-p53-His. Trình tự được tổng hợp nhân tạo, tích hợp trong vector pUC-TAT-p53-His và đã được chúng tôi ghép nối thành công vào vector biểu hiện pPICZαA ở nấm men *Pichia pastoris* bằng cách sử dụng đồng thời hai enzyme giới hạn *Eco*RI và *Xba*I tạo thành pPICZαA-TAT-p53-His. Sau khi được biến nạp vào *E. coli* và kiểm tra đúng trình tự ADN, vector tái tổ hợp này đã được biến nạp vào nấm men *P. pastoris* bằng xung điện. PCR với cặp mồi AOX1 cho thấy trình tự mã hóa TAT-p53-His đã dung hợp vào hệ gen của nấm men. Biểu hiện của protein tái tổ hợp ra môi trường ngoại bào đã được kiểm chứng bởi điện di SDS-PAGE.

Từ khóa: Pichia pastoris, biểu hiện gen, p53, cải biến mã bộ ba.