CLONING AND EXTRACELLULAR EXPRESSION OF RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR (RT-PA) USING A METHYLOTROPHIC YEAST PICHIA PASTORIS

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ABSTRACT

Tissue plasminogen activator (tPA) has noteworthy application in treatment of acute myocardial infarctions. This study focuses on expression of rt-PA using microbial systems in order to reduce cost without compromising on quality as an alternative to commercial (rt-PA)produced by using mammalian host systems. In the present study, Pichia pastoris X-33strain was used as a host with pICZαA expression vector to obtain extracellular expression of full length tPA gene. Specific primers were designed in such a way to get native tPA protein sequence in subsequent purification steps. Further, construct pICZαA-tPA was developed and electroporated into host to achieve stablert-PA gene by achieving genome integration. The transformants were screened for phenotypic characters.Mut^{+}phenotypic colony named Pichia tPA-3 showed expression of rt-PA at 66 kDa on SDS PAGE. Size Exclusion Chromatography (SEC) was performed, resulting in product peak at same RT as reference standard. (alteplase).Cloning and expression of rt-PA was successfully achieved in microbial system. Further process optimization at larger scales will surely provide cost effective alternative to mammalian system forrt-PA production.

KEYWORDS

Tissue plasminogen activator, acute myocardial infarctions, Pichia pastoris, pICZαA, FactorXa protease, SDS PAGE, Size Exclusion Chromatography etc.

1. INTRODUCTION

Proteins are being extensively used in therapeutics because of their high specificity and immunologically acceptability over small drug molecules. Globally, therapeutic proteins had a compound annual growth rate (CAGR) of 16.4% from 2002 to 2010 whereas, the market forecast is to grow at a CAGR of 6.2% from 2010-2017, to reach $141.5 billion in 2017. (GBI market report, 2011)

Therapeutic proteins are very popular because of their following advantages over small drug molecules: 1) Proteins often serve as a highly specific and complex set of functions that cannot be

mimicked by simple chemical compounds 2) The action of proteins is highly specific, there is often less potential for protein therapeutics to interfere with normal biological processes and cause adverse effects 3) The body naturally produces many of the proteins that are used as therapeutics these agents are often well tolerated and are less likely to elicit immune responses.The clinical development and FDA approval time of protein therapeutics may be faster than that of small-molecule drugs [8]. There are many examples of therapeutic proteins that have been successfully commercialized such as Insulin (Hormone), Factor VIII, IX (Blood coagulation factor), thrombolytic agents like Alteplase and Reteplase[17].

Tissue plasminogen activator (tPA) is an important component of fibrinolytic system to maintain the proper blood flow in the body. Thrombolytic therapy is a major treatment for cardio-vascular diseases such as acute myocardial infarction (AMI), cerebrovascular disease and deep vein thrombosis, are major causes of death and disability [22]. Tissue plasminogen activator is a serine protease having thrombolytic activity. It has advantage of causing no side effects such as systemic hemorrhaging and fibrinogen depletion over other types of plasminogen activators [14].Human tissue-type plasminogen activator is a 562 amino acid glycoprotein, having 67 KDa molecular weight. Tissue plasminogen activator consist of 17 disulfide bonds in 34 cysteine residues contributing to the characteristic folding of the chain. Structure of tPA protein comprises of five distinct domains: a fibrin binding 'finger' region, an 'epidermal growth factor' like sub domain, two disulphide looped 'kringle' domains and a carboxy-terminal serine protease 'catalytic' domain [18].

Initially, bowes melanoma cells were the primary source of tPA production for medical purposes. As the importance of therapeutics increased drastically, scientists made effortless experiments for cost effective abundant tPA production using mammalian and microbial host systems. However as time progressed, *Pichia pastoris* has emerged as the most compatible and successful host for expression of heterologous recombinant proteins. Now-a-days, more than 500 proteins have been cloned and expressed using this methylotrophic yeast system [5]. Several factors has contributed for its rapid acceptance as unique and highly useful over other microbial host systems; 1) a strong tendency for respiratory growth instead of fermentative growth, 2) ability to grow on minimal medium at very high cell densities, 3) as easy as *E. coli* for even genetic manipulation, 4)highly compatible to fermentation processes with no virus and endotoxins production, 5) secretes very minimal levels of host cell proteins and also avoids hyper-glycosylation [3]. But the most important characteristic of the *Pichia pastoris* is existence of very strong and tightly regulated promoter from alcohol oxidase 1 gene (PAOX1).

Alcohol oxidase is the first enzyme of the methanol assimilation pathway which catalyses the oxidation of methanol to formaldehyde[2]. There are two alcohol oxidase genes in *P. pastoris* which code for the alcohol oxidase enzyme, the alcohol oxidase 1 gene (AOX1), which is responsible for greater than 90% of the enzyme in the cell, and the alcohol oxidase 2 (AOX2) for less than 10%. There are three types of *P. pastoris* host strains available that vary with regard to their ability to utilize methanol. The wild type or methanol utilization plus phenotype (Mut^+), and those resulting from deletions in the AOX1 gene (methanol utilization slow Mut^s) or both AOX genes (methanol utilization minus Mut^-)[5].

In this study, for the first time *Pichia pastoris* X33 host cell system was used for cloning and expression of full length tissue plasminogen activator protein using novel primer design strategy.

2. MATERIALS AND METHODS

2.1. MATERIALS

Tissue plasminogen activator cDNA clone was purchased (Thermo Scientific open biosystems) and used as a source of gene for PCR amplification. *Escherichia coli* TOP10F' and *Pichia pastoris* X33 strains were used for cloning and expression of tPA gene respectively. Both strains were obtained from Pichia expression kit (Invitrogen, USA). PlasmidspTZ57R/T (Fermentas Inc. Vilnius, Lithuania) as cloning vector and pICZαA (Invitrogen, USA) as an expression vector were used in this study.Luria-Bertani media for *E. coli* and YPD media for *P.pastoris* were used to support growth of both strains (Hi-media Ltd, India). Buffered complex medium, containing glycerol (BMGY) was used for growing the host cells before induction, and buffered complex medium, containing methanol (BMMY) was used as an induction medium (Invitrogen, USA).

2.2. METHODS

2.2.1. Primers Design

Specially designed primers were used in this study. Both primers were designed with incorporation of restriction site.Additionally, forward primer named PPFortPA was also having FactorXa specific endo-protease recognition site (5'-ATTGAAGGTAGA-3'). Expression cascade of pPICZαA vector is represented in Figure 1. This vector has α factor secretion signal sequence which allows efficient secretion of recombinant protein from *Pichia* host cell. The C- terminal poly histidine tag permits purification of protein on metal-chelating resin. Restriction enzyme *Xho I* and *Xba I* were selected from MCS and restriction sites incorporated in PPFortPA and reverse primer named PPRevtPA respectively. This design helped to maintain open reading frame of the gene after successful cloning into vector. After expression, during polishing steps, use of FactorXa protease enzyme facilitates cleavage at C-terminal of Arginine present in recognition site eventually providing the native tissue plasminogen activator protein with no unwanted amino acid stretch.

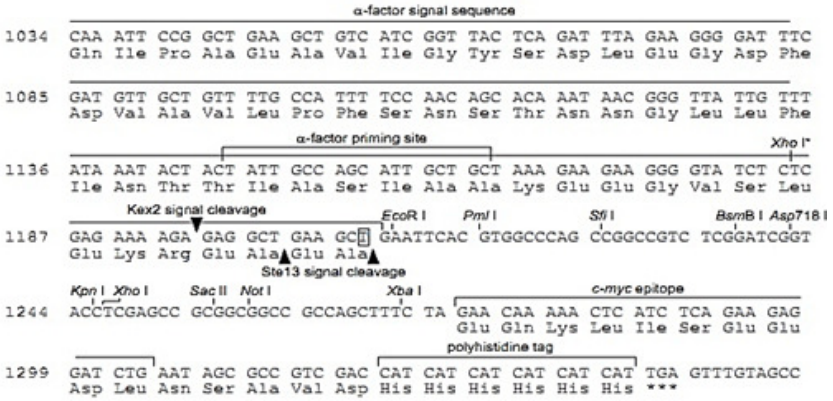

Figure 1: Expression cascade of pPICZαA vector which includes α-factor secretion signal sequence originally from saccharomyces cerevisiae, multiple cloning site (MCS) facilitating cloning and C-terminal tags for ease of purification.

2.2.2.PCR amplification and cloning of tissue plasminogen activator gene

Tissue plasminogen activator cDNA clone (Gene bank accession number BC013968.2) was used as PCR template (source of GOI) while, PPFortPA (5'-CTCGAGATTGAAGGTAGAATGGATGCAATGA AGAGAGGGCTC -3') and PPRevtPA (5'-TCTAGAACTCACGGTCGCATGTTGTCACGAATCCAGT CTAG -3') were used to amplify full length tPA gene. The tPA gene was amplified using high fidelity PCR (Thermal cycler C1000, Bio-Rad Laboratories, USA). All PCR reactions were subjected to 34°C thermal cycles of denaturation for 1 min at 95°C followed by temperature gradient for annealing at 64°C to 71°C for 1 min and then extended for 2 min at 72°C, with initial 1 cycle of denaturation for 5 min at 95°C and final extension for 10 min at 72°C. Then, 1710 bp PCR product was further purified using Pure link- PCR purification kit (Invitrogen, USA) and purified amplicon (PCR product) was confirmed by restriction enzyme analysis done using 1% agarose gel electrophoresis.

PCR amplification of tPA gene was performed using Taq-DNA polymerase which has an advantageous terminal transferase activity. This enzyme adds a single 3'-A overhang to both ends of the PCR product. This structure favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Using this principle, purified amplicon was ligated with pTZ57R/T cloning vector maintaining GOI to vector ratio 3:1 as per below formula;

$$Concentration\ of\ GOI\ (ng) = \frac{Concentration\ of\ Vector\ (ng) \times size\ of\ GOI\ (Kb)}{Size\ of\ Vector\ (Kb)}$$

Thus, an intermediate construct pTZ57RtPA was developed using InsT/Aclone PCR cloning kit (Fermentas Inc. Vilnius, Lithuania). Construct was transformed successfully into *E. coli* TOP10F' and transformants were screened using blue white screening technique. Restriction mapping was also performed to check the correct orientation of the insert into an intermediate vector of positive transformants. Further confirmation was done by performing bidirectional sequencing of the intermediate construct.

Preparation of final construct was started with restriction digestion of both pTZ57RtPA and pICZαA vectorusing *Xho I* and *Xba I* enzymes. This step provided full length tPA gene with FactorXa protease recognition site on N-terminal available for cloning into expression vector. Thus, Final construct was developed and named as pICZαAtPA. Successful cloning was confirmed by three distinctive test such as; 1) Colony PCR of screened transformants, 2) Restriction analysis using same set of restriction enzymes and 3) Bidirectional sequencing was performed using primers 5'AOX promoter (5'- GACTGGTTCCAATTGACAAGC-3') and 3'AOX terminator (5'-GCAAATGGCATTCTGACATCC-3') to ensure reading frame of the gene prior to expression.

2.2.3.Transformation of construct into Pichia pastoris X33 cells

Nucleotide sequence of final construct pICZαAtPA was subjected again to NEB cutter web tool analysis which resulted in to use *Pme I*restriction enzyme for linearization of construct. This enzyme provides a single cut at 414 bp into AOX promoter region to linearize the construct. This linearized construct upon transformation, integrates into *Pichia pastoris* genome by homologous recombination at AOX1 promoter locus thus providing stability to insert gene of interest. Five µl

of *Pme I*restriction enzyme was used to achieve complete linearization of the construct. Further chemically competent *Pichia pastoris* X33 cells were prepared using material and procedure provided into *Pichia* expression kit (Invitrogen, USA). Linearized construct pICZαAtPA was transformed into the active cells using standard protocol provided by the manufacturer (Invitrogen, USA). Transformation reactions were spread and allowed to grow on YEPD agar plates containing 100 µg/ml zeocin antibiotic. Colony PCR was performed to confirm the transformation and stability of final construct using a pair of primers of AOX promoter locus.

Homologous recombination of AOX promoter locus of linearized vector with *Pichia* genome can provide two possibilities; either the AOX1 promoter locus will remain intact representing Mut^+ phenotypic character (Methanol utilization plus), or theAOX1 promoter locus gets disrupted resulting in pseudo AOX1 i.e. AOX2 promoter which will be represent Mut^S phenotypic character (Methanol utilization slow). Thus, after successful transformation of construct into *Pichia pastoris* X33 host cells, screening of positive transformants were done for phenotypic character determination. Transformants were streaked on both minimal dextrose with histidine (MDH) and minimal methanol with histidine (MMH) agar plates. Plates were incubated at 30°C for minimum 3 days and growth of isolated colonies was monitored.

2.2.4.The expression of recombinant Pichia pastoris X33 strain

Single colony was inoculated in 100 ml of BMGY medium, incubated at 30°C in a shaker incubator at 230 rpm. The cells at OD600 = 4 were harvested by centrifugation at 2000 ×g for 5 min at RT and re-suspended in 200 ml of BMMY medium for induction at 30°C. Every 24 hours, 100% methanol to a final concentration of 0.5% was used to maintain the induction phase till log 144 h.

2.2.5.SDS PAGE and Size Exclusion chromatography

Perday 1sample were collected from age of 24 h,48h, 72h, 96h, and 120htill 144 h and centrifuged to get the supernatant required for further expression analysis. Analysis was done by 10% SDS PAGE with positive control reference standard tPA protein (Alteplase) against pre stained protein molecular marker. Same set of samples were further injected sequentially into a size exclusion chromatography (SEC) system in comparison to a reference standard tPA protein (Alteplase). Samples were filtered with 0.22 µm filter and 5 µl sample volume was injected for each sample. System was run at 0.75 mL/min flow rate at room temperature with run time of 60 minute each.

3. RESULTS

3.1. CLONING OF TISSUE PLASMINOGEN ACTIVATOR

Restriction mapping analysis of pTZ57RtPA showed a successful cloning of the full length tPA gene into cloning vector with correct orientation. Full length tPA gene was subjected to NEB cutter tool web tool analysis resulting in use of *SacI* restriction enzyme. Itcutat only one site in between tPA gene sequence at 1337/1341 bp providing two distinct bands of 1.3 Kb and 400bp when analyzed on agarose gel electrophoresis. Thus, it is prominent from the results that the concept of restriction mapping was used

successfully to identify the intermediate clone with a correct orientation of the tPA gene (insert) into vector (Figure 2).

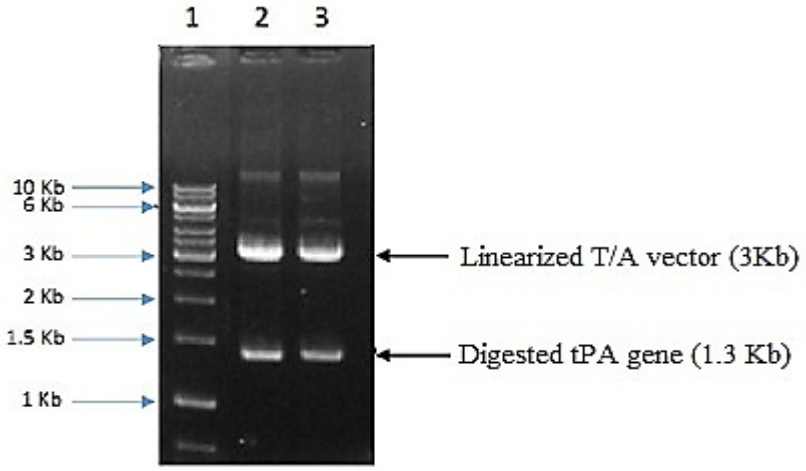

Figure 2Agarose gel electrophoresis of restriction digestion of PCR positive recombinants, Lane 1; 1 kb DNA molecular marker, Lane 2 & 3; PCR positive recombinants

After Restriction mapping of intermediate construct, sub cloning of tPA gene into expression vector was achieved. Colony PCR was performed for positive transformants of pICZαAtPA expression construct, which showed successful ligation of tPA gene into pPICZαA expression vector (Figure 3). All 6 positive transformants (colonies) selected showed PCR amplicon of 1.7 Kb comparing with (positive control) reference tPA cDNA PCR amplicon and molecular marker.

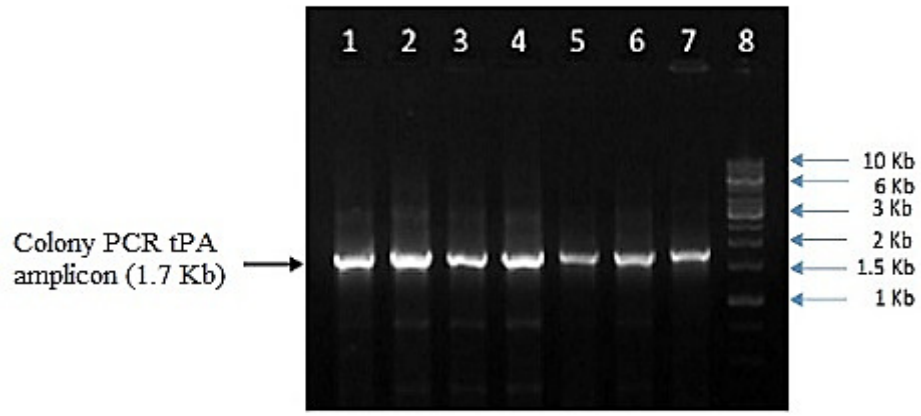

Figure 3Agarose gel electrophoresis of Colony PCR of tPA-pICZαA transformants, Lane 2; PCR positive control, Lane 8; 1 kb DNA molecular marker and Lanes 1, 3-7; Colony PCR positive tPA- pICZαA transformants

Furthermore, as 2nd distinctive test, restriction analysis of final construct pICZαAtPA reflected successful sub cloning of tPA gene from cloning vector to expression vector. The size of final expression construct was 5303 bp and digestion of this construct with *Xho I* and *Xba I* restriction

sites resulted into two different fragments of linearized pICZαA vector (3593 bp) and tPA clone (1710 bp) respectively compared with (negative control) expression vector without insert. (Figure 4)

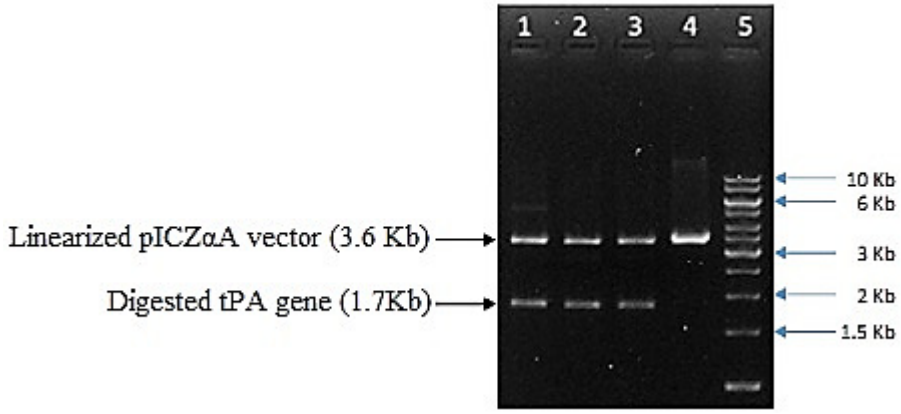

Figure 4Agarose gel electrophoresis of restriction digestion of tPA-pICZαA PCR positive recombinants, Lane 1-3; tPA- pICZαA Colony PCR positive recombinants, Lane 4; pICZαA vector w/o insert, Lane 5; 1 kb DNA molecular marker.

Bidirectional sequencing was performed as 3rd distinctive test. NCBI blast of both forward and reverse primer query sequences with reference tPA cDNA clone sequence (Gene bank accession number BC013968.2) proved successful cloning of full length tPA gene into an expression vector maintaining it's open reading frame such as to express recombinant tPA protein with native sequence. Sequencing resulted with 100% similarity with the reference, thus finally delivering desired pICZαAtPA expression construct. Thus after these tests it was proved that, pICZαAtPA expression construct was developed successfully with correct orientation of the insert into vector maintaining open reading frame for protein translation.

3.2. TRANSFORMATION OF CONSTRUCT

Prior to transformation, expression construct was linearized using *Pme I* enzyme and transformed successfully into chemically competent *Pichia pastoris* X33 cells. Further, experiment to identify phenotypic character was conducted. Isolated colonies on MMH plates were observed resulting in Mut^+ phenotype of *Pichia pastoris* X33 cell containing pICZαAtPA construct. Positive transformants were screened and colony named *PichiatPA3*was further taken up for expression analysis.

3.3. SDSPAGE AND SEC ANALYSIS

Daily samples collected from expression study were centrifuged before SDS PAGE analysis. In Figure 5, Lane 1 to 4 are samples collected at 48 h, 72h, 96h and 120 h respectively. Lane 5 represents to pre-stained protein standard molecular marker (Bio-Rad Laboratories, USA) while lane 6 is showing band of positive control (Alteplase – commercial recombinant tPA protein, at 66kDa). Thus in SDS PAGE analysis, expression of recombinant tPA protein was observed successfully compared with positive control and pre stained protein molecular marker.

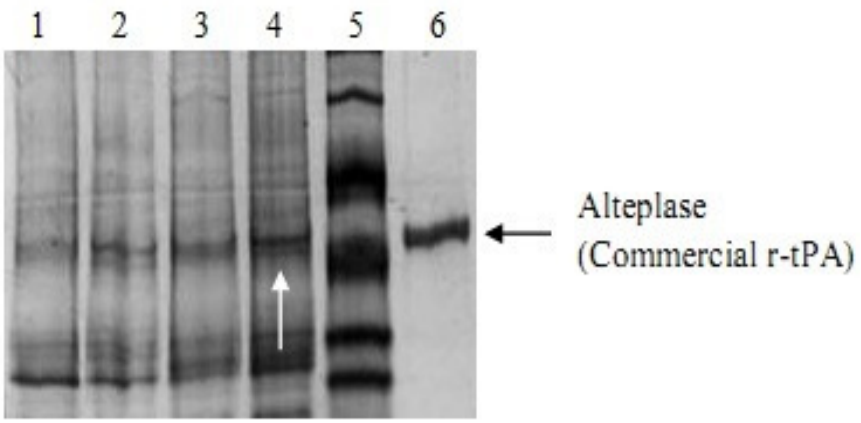

Figure 5tPA expression analysis using SDS PAGE; Lane 1 to 4: are samples drawn at 48h, 72h, 96h, and 120h respectively. Lane 5: Pre-stained standard protein molecular marker, Lane 6: Positive control (Alteplase).

Further size exclusion chromatography was performed for the same set of samples. Sample at 120 h showed same retention time as of positive control i.e. standard commercial r-tPA (Alteplase) at 7.6 min. similar pattern was observed for both samples during chromatographic run, thus concluding successful expression of full length recombinant tissue plasminogen protein using *Pichia pastoris* X33 cells.

4. DISCUSSION

Tissue plasminogen activator is one of the important therapeutics with growing demand in biotechnology market. Initially, tPA produced from bowes melanoma was costing $22000 for 100 mg dosage which was very costly and limited too. Thus, use of mammalian host system was started for tPA production. Tissue plasminogen activator is the first therapeutic protein been commercialized and produced using recombinant CHO cell lines. As per current market scenario, Alteplase (r-tPA) produced by recombinant CHO cells cost approximately $350 per 20 mg of dosage (Boehringer Ingelheim, Germany). Production cost of therapeutics with use of mammalian host system is very high compared to microbial hosts, thus efforts were taken to produce tPA using microbial host systems [19].

The enteric bacterium *Escherichia coli* is one of the most extensively studied and used prokaryotic organisms for the commercial production of therapeutic proteins. Compared with other established expression systems, *E. coli* offers several advantages, including growth on inexpensive carbon sources, rapid biomass accumulation, amenability to high cell-density fermentations, simple process scale-up and availability of large number of cloning and expression vectors along with respective host strains. Considering these advantages, various attempts were made to produce tPA using *E. coli* resulted to identify requirement of disulfide bond formation machinery. Thus, first attempt to produce full length tPA was done by co-overexpressing enzymes which supported disulfide bond formation into cytoplasm [13]. But co-overexpression lead to formation of inclusion bodies, thus extracellular secretion of only biologically active part of tPA (K2S domain) was further attempted by researchers. OmpA secretion signal was used and

approximately 68% recombinant K2S secreted in supernatant found to be active [14]. Reteplase - a deletion mutein of tPA was also produced by *E. coli*, but major disadvantages of this form was its weaken affinity for fibrin compared to full length tPA, causing more fibrinogen depletion thus resulting in higher frequency of bleeding complications [22].

However, native human gene expression in bacterial systems require extra cellular machinery for post translational modifications (PTM). Also bacteria produce endotoxins and there is potential for protein degradation[18]. To overcome such problems yeasts have been focused upon. These organisms do not produce endotoxin and are capable of performing 'Post Translational Modifications' (PTM) of proteins up to a certain extent like mammalian cells so as to deliver functional recombinant proteins. They are easier and less expensive to work with than insect or mammalian cells, and are easily adapted to fermentation processes. Yeasts such as *Saccharomyces cerevisiae, Hansenullapolymorpha,* and *Pichia pastoris* are among the simplest eukaryotes that have been used for the production of FDA-approved therapeutic proteins [6].
Attempts were also made using *Aspergilusniger*[12], *Aspergilusnidulans*[20]but heterogenicity were observed in the produced recombinant tPA protein. *Saccharomyces cerevisiae*[8],[15] used for r-tPA production lead to hyperglycosylation and despite presence of native yeast secretion signal produced r-tPA was found to be intracellular leading to cell disruption for product recovery.

In last two decade, researches have focused on *Pichia pastoris* to use as host for heterologous proteins production. *Pichia* has been used to successfully produce proteins across a broad spectrum of functional types, including enzymes, antigens and engineered antibody fragments, for example: human angiogenin[11], xylanase, rHSA fusion protein [23], rIL-3[10] and many more.
Therefore in present study,*Pichia pastoris* was used as host for r-tPA production. Specific primers were designed using *FactorXa* protease recognition site incorporated in forward primer which would help in finishing steps of purification. *FactorXa* cleaves protein at specific recognized site removing unwanted amino acid stretch delivering native tPA protein sequence. Earlier literature has reported on expression of tPA using *Pichia pastoris* GS115 with three extra amino acids at N-terminus of protein by researchers [12]. This might interfere with proteins function or can also explicit allergic responses in body. Such problems were addressed by designing specific primers with incorporated *FactorXa* protease coding site.

Full length tPA gene was amplified using specially designed primers and Purified PCR amplicon was cloned into pICZαA expression vector. Native α-factor secretion signal sequence incorporated into vector allows extracellular expression of protein. The expression construct *pICZαAtPA* was linearized and transformed *Pichiapastoris* X-33 strain to achieve genome integration and stability of tPA gene. Isolated colonies grown on zeocin containing medium were screened for positive transformants. *Pichiapastoris* X-33 is a wild type strain that has both AOX1 and AOX2 promoter locus intact to achieve higher levels of expression with methanol induction. *Pichia pastoris* GS115 is a mutated strain in histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. Positive transformants were screened for Mut^+ or Mut^S phenotypic characters. Among, the 24 positive transformants screened, 10 were found to be Mut^+. One of the colonies named *PichiatPA3* showed expression at 66 kDa on SDS PAGE after 120 hrs of induction with 0.5% v/v of 100% methanol. Previously, scientists have also cloned and expressed full length tissue plasminogen activator in *Pichia pastoris* GS115 strain which is His^- mutant and transformants obtained was Mut^s [12].

Here in this study, we represented successful cloning and expression of Recombinant tPA protein using *Pichia pastoris X33 strain*.Enzyme activity quantification of recombinant tissue plasminogen activator protein needs to be done and further process optimization at larger scales will surely provide a cost effective alternative to use of mammalian system for rt-PA production.

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