CHAPTER 2

EXPRESSION OF ANTI-TNFα scFv (RECAB) IN YEAST

(*Pichia pastoris*)

INTRODUCTION

The advantage of recombinant DNA technology is the industrial scale production of pharmaceutically important proteins, whereas purification from natural sources is unfeasible and uneconomical. Functional and structural properties of the protein produced in small quantities in their natural sources can be investigated by producing them in the heterologous hosts. Production of novel protein has also been made feasible by application of protein engineering and expression of these proteins in other host systems. At present, there are more than 100 biotechnology derived therapeutics and vaccine approved by Food and Drug Administration (FDA), USA for medical usage and many more drugs and vaccines are in various phases of clinical trials (Butler et al., 2014; Lawren, 2015).

Escherichia coli has been the host of choice for recombinant protein production for more than two decades. The relatively high growth rates of *E. coli* and its ability to grow to high cell density has led to extremely high volumetric productivities (Hewitt and McDonnell, 2004). A large number of recombinant proteins are being produced industrially using *E. coli* as a host system. In spite of the early success in the production of heterologous protein in *E. coli*, researchers began to exploit alternative host system for the expression of eukaryotic proteins. The key reasons behind this were that *E. coli* being a prokaryotic system lacked the ability to perform the important functions such as RNA splicing, post-

translational modification, and secretion of the foreign protein which are restricted to eukaryotic organism (Duilio et al., 2004). When proteins are produced intracellularly at high levels, the amount of soluble active protein present is usually limited due to the formation of inclusion bodies. The protein in the inclusion body is often misfolded. Resolubilization of inclusion bodies is tedious and the yields are often low. If the product is produced intracellularly, the cell must be lysed for protein recovery which results in the release of endotoxins (or pyrogens) from *E. coli* (Liljequist and Stahl, 1999; Hansson et al., 2000).

Among the microbial eukaryotic host system, yeasts enjoy advantages of both prokaryotic and eukaryotic systems. It is easy to manipulate at molecular genetic level, produce protein both extracellularly and intracellularly, and it perform eukaryotic protein modification such as glycosylation, disulfide-bond formation, and proteolytic processing (Cregg et al., 2000, Eckart and Bussineau, 1996).

Foreign proteins expressed in yeasts are not only being used to synthesize life saving drugs in the pharmaceutical industries, but also to unravel the complex regulatory phenomena in basic research. The major advantages include; ease with which it can be grown in chemically defined media, genetic manipulation techniques available and absence of endotoxin as well as oncogenic or viral DNA. *Saccharomyces cerevisiae* was among the first and most commonly used eukaryotic expression systems. It has also been used for the production of bread

and beer since long time, so it has been recognized by the US Food and Drug Administration (FDA) as an organism generally regarded as safe (GRAS). Compared to the other complex eukaryotic expression systems, such as Chinese hamster ovary cell and baculo virus-infected cell lines, yeasts are economical, usually give higher yields and are less demanding in terms of time and effort (Cregg, 1999).

Since early 1980's, production of vaccines and several other pharmaceutically important products were initially restricted to *S. cerevisiae*. Although, well characterized*, S. cerevisiae* has certain limitations, such as low production yields even with strong promoters, hyper glycosylated protein and intracellular or periplasmic retention with consequent partial degradation of proteins larger than 30 kDa (Cereghino et al., 2002). Therefore, to overcome these limitations, several new expression systems have been developed. These include the methylotrophic yeasts *P. pastoris* and *Hansenula polymorpha,* alkane-utilizing yeast *Yarrowia lipolytica,* xylose-fermenting yeast *Pichia stipitis*, starch-fermenting yeast *Schwannionmyces* (Debaryomyces) *occidentalis* and the lactose-fermenting yeast *Kluveromyces lactis.*

Pichia Pastoris is methylotrophic yeast, which grows to very high cell densities in chemically defined media. Using *P. pastoris*, high expression level (5-40% of cell protein) were achieved, relatively much higher than baker's yeast, and often

equivalent to *E. coli* or baculovirus system (Romanos et al., 1992). Additionally, scale-up of *P. pastoris* culture is simple and leads to enhanced protein yields on a volumetric basis, (14.8 g/L) of gelatin secreted in recombinant *P. pastoris* (Werten et al., 1999).

There are many advantages of using this host for expression of heterologous proteins. *P. pastoris* is fast growing and can grow to higher cell densities of 100g/L (dry weight), which are hard to achieve with *S. cerevisiae* (Cereghino et al., 2002). It posses strongest most regulated promoter, (AOX1), enabling the level of product expressed to be regulated by a simple manipulation of the culture medium. The post translational modification machinery of *P. pastoris* is very efficient and has enabled the production of several functional mammalian glycoproteins, which are essentially indistinguishable from the native product (Abdulaev et al., 1997). Additionally, *P. pastoris* derived product has no problem of contamination with endotoxins as in the case of bacterial systems, giving it an extra advantage for the production of vaccines or therapeutic drugs (Joosten, 2003). Genome sequence of strain CBS7435 (Küberl et al., 2011) and GS115 (De Schutter et al., 2009) has been published; genome sequences and gene annotation will facilitate the strain engineering, identifying new promoters and progressing in the biology *of P. pastoris.*

A number of therapeutic proteins has been successfully expressed and commercialized using *Pichia* as an expression system. Examples are: Plasma kallikrein inhibitor (Kalbitor® (ecallantide)) indicated against hereditary angioedema produced by Dyax (Cambridge, MA, USA), Jetrea®, used for treatment of symptomatic vitreomacular adhesion was approved by the FDA and the European Commission, insulin produced by Biocon, Elastase inhibitor against Cystic fibrosis and many more (Gonçalves et al., 2013). Since the first report of the expression of antibody in *P. pastoris*, number of attempts has been done to optimize glycosylation and expression level (Li et al., 2006). Li et al., (2006) produced antibody in glycoengineered *P. pastoris* strain and showed that the produced antibodies has similar glycosylation pattern to that of human. GlycoSwitch and GlycoFi technologies have optimized the glycoengineered antibody production up to 1.6 g/l (Potgieter 2009; Ye 2011).

Single-chain variable fragments (scFv) are small recombinant antibodies fragments consist of the variable domains of the light and heavy-chain (V_L, V_H) joined together with a flexible peptide linker (Huston 1988). There are many advantages in using scFvs; they retain the binding affinity and specificity of their parent immunoglobulins, easier to manipulate and can be easily expressed in different expression systems. *P. pastoris* has also been proven to be a powerful candidate for

high level expression of functional scFv fragments with reports of yields ranging from 50 mg up to 4.88 gram per liter of culture (Damasceno et al., 2004; Freyre et al., 2000; Fischer et al., 1999; Khatri and Hoffmann, 2006).

Tumor necrosis factor alpha (TNFα), also called as fire alarm of immune system, is a cytokine involved in systemic inflammation. Cells like monocyte/macrophage lineage with T-lymphocyte, neutrophils, mast cells and endothelium cells are major sources of TNFα. Large amount of TNFα is released in response to lipopolysaccharide and other bacterial products. Dysregulation and, in particular, overproduction of TNF have been implicated in a variety of human diseases like autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis and so on. TNFα inhibitors (monoclonal antibodies and fusion proteins) are used to treat these diseased conditions. The generation of monoclonal antibodies as therapeutics emerged as a high demand clinical need to meet the growing requirement of autoimmune disease therapy.

scFv protein which has a simple structure can be produced in *E. coli* cells. The expression level of these bacterial expressed scFv is low when expressed extracellularly and intracellularly they are not amenable for utilization as they

often produces inclusion bodies they renature inefficiently and showed poor solubility (Denzin and Voss, 1992; Mallender et al., 1996). ScFv expression and insolubility can be overcome by expressing in the methylotrophic yeast *P. pastoris*. Considering the merits of *P. pastoris* as an expression system, present study focuses on the expression of anti hTNFα scFv (Recab) in this system.

Objectives

1. Expression of Recab protein in *P. pastoris*
2. Shake flask study for Recab expression
3. Functional assay of Recab protein on HeLa cell line

2. MATERIALS AND METHODS

2.1 Strains and culture conditions

Bacterial and yeast strains used in the present study are described in appendix Table 3. Bacterial transformations and plasmid propagation were carried out in *E. coli* strain DH5α, according to standard protocol (Sambrook et al., 1989). Yeast cultures were grown and maintained in YPD at 28^{0}C.

2.2 Transformation of *E. coli* with plasmid DNA

Competent cells of *E. coli* were prepared using calcium chloride method (Sambrook et al., 1989). Single colony of *E. coli* DH5α was inoculated in 3 ml of Luria-Bertani broth and grown overnight. 100 ml of fresh LB broth was inoculated with 1 ml of overnight grown culture and grown at 37^{0}C up to 0.3 to 0.4 O.D$_{600}$; chilled on ice and the cells were pelleted by centrifugation at 2012 g for 5 min at 4^{0}C. The cells were re-suspended in ice-cold 0.1 M $CaCl_2$ and incubated for 30 min. The cells were re-harvested by centrifugation and re-suspended in ice-cold 0.1 M $CaCl_2$ with 20% glycerol. The culture was stored at -70^{0}C in 100µl aliquots. For each transformation, a 0.1 ml aliquot of this cell suspension was transferred to a 1.5 ml chilled microfuge tube; mixed with the plasmid DNA (usually 50 ng) or ligation mixture and incubated at 4^{0}C for 30 min. Cells were subjected to heat shock at 42^{0}C for 90 sec, followed by 5 min. incubation on ice. 1 ml of LB broth was added to the above suspension and incubated at 37^{0}C for 30 min. About 0.1 ml

aliquot of transformation mixture was plated on appropriate selection media containing either ampicillin (100µg/ml) or kanamycin (50µg/ml).

E. coli cells transformed with the ligation mixtures were tested for α complementation to identify bacterial colonies that contain recombinant plasmid, wherever it was applicable (Sambrook et al., 1989). The blue and white color selection was carried out by addition of 30 µl of X-gal (stock- 20µg/ml in dimethyl formamide) to 30 ml of LB agar plates containing ampicillin. The white colonies obtained were further analyzed by carrying out mini-preparation of DNA.

2.3 Isolation of plasmid DNA

2.3.1 Small scale plasmid DNA isolation

Plasmid DNA was isolated from the recombinant *E. coli* cells using CTAB method (Del et al., 1989). Overnight grown culture (1.5 ml) was centrifuged in a microfuge tube and the bacterial pellet was re-suspended in 200 µl of STET. Lysozyme (25µl) was added to it, mixed by vortexing and boiled in a water bath for 45 sec followed by centrifugation at 13414 g for 10 min. The resulting snot was removed with a tooth pick and 20 µl of 5% CTAB was added followed by centrifugation for 5 min. To the pellet, 300 µl of 1.2 M NaCl and 750 µl of 100% ethanol was added. The DNA pellet was obtained by centrifugation for 10 min; washed with 70% ethanol and re-suspended in 50 µl of TE. The yield of plasmid DNA obtained was around 5-7 µg.

2.3.2 Medium scale isolation of plasmid DNA

Medium scale preparation of plasmid DNA was carried out as follows: cells were grown overnight in 50 ml of LB broth, pelleted by centrifugation at 6238 g for 5 min, washed with 2 ml of TE (50 mMTris pH 8.0, 1 mM EDTA pH 8.0). One ml each of cells was transferred to two microfuge tubes; centrifuged at 13414 g for 30 seconds and re-suspended in 300 µl of STET (15% sucrose, 50 mMTris pH 8.0, 1 mM EDTA pH 8.0). Lysozyme solution (100 µl of 10 mg/ml) was added and the cells were incubated on ice for 10 min. This was followed by addition of 370 µl of TTE (0.1% Triton X-100, 50 mMTris pH 8.0, 50 mM EDTA) and the cells were incubated for 10 min after mixing the contents thoroughly. Cells were boiled for 1 min in a water bath, centrifuged at 13414 g for 10 min to remove the snot, and the supernatant was precipitated with 2 volumes of ethanol (room temperature). The DNA was collected by centrifugation at 13414 g for 5 min and each pellet was dissolved in 250 µl of water. The DNA was treated sequentially with RNAse (20 µl of 1mg/ml stock) and Proteinase K (10 µl of 10 mg/ml stock), extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with two volumes of chilled ethanol. The purified DNA pellet was obtained by centrifugation at 13414 g for 10 min, washed with 70% ethanol and suspended in 100-150 µl of TE (10 mMTris pH 8.0, 1 mM EDTA pH 8.0). The total yield of the plasmid DNA extracted using this method was 150-250 µg.

2.4 Nucleic acid manipulations

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended by the suppliers. Ligations were carried out at 20^0C, overnight for blunt ended DNA fragment and at 16^0C for 3-4 h for sticky end fragments.

2.5 Transformation of *Pichia pastoris* using LiCl method

Transformation of *P. pastoris* strains X33 and GS115 *his4*$^-$ *mut*$^+$ was performed by lithium chloride method as described in the "*Pichia* expression Kit". A single yeast colony was inoculated in 10 ml YPD medium and incubated overnight at 200 rpm. This culture (500 µl) was inoculated in 50 ml of fresh YPD medium and incubated at 30^0C with shaking until an OD_{600} of 1.0 was reached that corresponded to a cell density of approximately 10^8 cells/ml. Cells were washed three times with sterile water and re-suspended in 1 ml of 100mMLiCl. Cells were briefly centrifuged and re-suspended in 400 µl of 100 Mm LiCl. For transformation, 50 µl cells were taken and briefly centrifuged, to the pellet transformation mix (240µl 50% PEG, 36µl 1 M LiCl, 25µl 2 mg/ml single stranded DNA and 5µg plasmid DNA linearized with *Pme*I) was added. It was mixed thoroughly and gently and was incubated at 30^0C for 30 min without shaking. This was then heat shocked at 42^0C for 20 min. Mixture was centrifuged and then cells were re-suspended in 1 ml YPD ad incubated at 30^0C with shaking. After 1 and 4 h, 100 µl aliquot was spread on YPD

zeocin (100µg/ml) (Invitrogen Life Technologies, California, USA) agar plate. The transformants were identified after 3-4 days.

2.6 Transformation of *P. pastoris* by electroporation

Transformation of *P. pastoris* strains GS115 and X-33 was performed following a standard protocol (Becker and Guarente, 1991). A single colony was inoculated in 1 ml YPD medium and incubated overnight at 200 rpm, 30^0C. This culture (100 µl) was inoculated in 100 ml of fresh YPD and incubated overnight at 30^0C and 200 rpm until a OD_{600} of 1 was reached, corresponding to a cell density of 10^8 cells per ml. Cells were washed three times and finally re-suspended in 1 ml of 1M sorbitol. 80 µl of these electro-competent cells were mixed with 1-2 µg of linearized plasmid and the end volume was adjusted to 100µl. The cells were incubated for 10 min on ice, transferred to a 0.2-cm electroporation cuvette and the subjected to electroporation (5 mS, 1.5kV, 25µF and 200W electroporation pulse) using a gene pulser (Bio-Rad, Hercules, CA, U.S.A.). The cells were diluted with 1 ml of 1M sorbitol and transferred in 15 ml sterile tube and incubated for 1-2 h at 30^0C without shaking. 100µl aliquots were spread on YPDS (YPD +1M sorbitol) agar plate containing 100 µg/ml zeocin.

2.7 Yeast Genomic DNA Isolation

Genomic DNA was extracted from yeast following the method of Hoffman and Winston (1987). Cells from exponentially growing yeast culture in a total volume of 50 ml were harvested by centrifugation at 5000 g for 5 min at room

temperature. The cell pellet was washed twice with 10 ml of TE. The pellet was re-suspended in 7ml of yeast extraction buffer along with 5g of glass beads (3mm diameter) and 7 ml of phenol: chloroform: isoamyl alcohol mixture (25:24:1). The mixture was vortexed for 5 min at maximum speed. The lysate was centrifuged at 13,000 g for 10 min at room temperature. Supernatant was removed in another tube to which 0.6 volumes of isopropanol was added. DNA was precipitated by incubating the mixture at room temperature for 30 min followed by centrifugation at 13,000 g for 10 min at room temperature. The DNA pellet was air dried and suspended in 1 ml of TE. 10 µl RNAse (stock- 1mg/ml) was added to each tube and incubated at 65^0C for 30 min followed by the addition of Proteinase K (stock- 2mg/ml)and further incubated at 50^0C for 30 min and extracted once with phenol: chloroform: isoamyl alcohol mixture (25:24:1). The final pellet of DNA, after precipitating with 2 volumes of ethanol was re-suspended in 250 µl of TE.

2.8 Southern hybridization analysis

Genomic DNA (2µg) from untransformed and transformed yeast was digested to completion with restriction endonuclease as recommended by the supplier; electrophoresed on 0.8% agarose gel, denatured (0.5 M NaOH for 30 min), neutralized (25mM sodium phosphate, pH6.5) and transferred onto nylon membrane (Hybond $N^{+,}$ Amersham, Buckinghamshire, England). DNA was fixed to the membranes by UV cross-linking ($12x10^4$µJ/cm2) using UV cross-linker.

Hybridisation probe was labelled non-radioactively using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer's instructions (Amersham Biosciences, Buckinghamshire, England). Membranes were exposed to X-ray films with intensifying screen for appropriate time at -70^{0}C. Standard procedures were followed for autoradiography.

2.9 Total RNA extraction and RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

The yeast strains were grown to mid log phase (OD_{600} of 1.0). Cells were centrifuged at room temperature (5000 rpm for 5 min). The supernatant was discarded and pellet was used for RNA extraction. Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, California, USA) as per supplier's instructions. The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. 5 µg of total RNA was used to synthesise the first strand cDNA using MuMLV reverse transcriptase (Fermentas GmBH, St. Leon-Rot, Germany) and oligo $(dT)_{12}$ in 20µl reaction system. Five microlitre of this RT product was used to carry out the RT-PCR.

2.10 Generation of Recab expression construct

The strategy used for generation of Recab expression construct (Recab Concentamer) is schematically illustrated in **Figure 1**. Briefly, coding region of Recab (750bp) was amplified using ATNF and ATNR primers (appendix table 3)

from pITC2 phagmid. The PCR amplified Recab gene was digested and cloned at the *Cla*I and *Xba*I site of pPICZαC vector, in frame with α factor signal sequence. The resulted vector, pPIC-Recab expression construct, was used further to generate vector with two copies of Recab named as Recab concentamer, following the *in vitro* multimerization procedure of Brierley, 1998. In brief, pPIC-Recab expression construct was digested with *Bgl*II-*Bam*HI and released Recab cassette, which was further cloned at unique site of *Bam*HI of pPIC-Recab expression construct, which already contained a single copy of the Recab expression cassette (**Figure 1**). The resultingvector, Recab concentamer thus had two copies of the Recab expression cassette in head to tail configuration and retained a unique *Bam* HI site at the end of the expression construct. Recab expression construct and Recab concentamer were digested with *Pme*I and linear vectors were used to transform *P. pastoris* strains X33 and GS115. The resulted transformants had Mut^+ phenotype and grew efficiently on methanol containing medium. Transformants were selected on YPD medium containing zeocin at final concentration of 100µg/ml which were further confirmed using PCR followed by Southern blot analysis.

2.11 Screening of Recab transformants of *P. pastoris* using PCR

The putative Recab transformants of *P. pastoris* were screened for the integration of the expression cassette. Screening of transformants was carried out by colony

PCR using α factor F and c-myc R primers. pPICZαC vector was used as a negative control.

2.12 Expression of Recab in *P. pastoris* transformants

For expression of Recab, *Pichia* transformants were grown in 100 ml capacity Erlenmeyer flasks containing 10 ml BMGY medium. The cultures were grown at 30^0C in a shaking incubator at 200 rpm until the culture reached an OD_{600} of 2-6. Cells were harvested by centrifugation and were re-suspended to an OD_{600} of 1.0 in BMMY medium (100 ml Medium in 1L capacity Erlenmeyer flasks). In order to maintain induction, methanol was added after every 24 h to raise its concentration in broth to 1 % (v/v). Samples were collected at an interval of 12 h and centrifuged at 10,000 g for 5 min. Supernatant were analysed on 12% SDS-PAGE to check the expression of Recab.

2.12.1 Western blot analysis

The protein samples were electrophoresed on 12% SDS- polyacrylamide gels, followed by electro transfer to nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England). The membrane was incubated in the blocking solution (3 % BSA, 0.2 % Tween 20 and 1x PBS) for 2 h. The blocking solution was discarded and the membrane was incubated with primary antibody (for Recab, Protein-L HRP and for TNFα, recab) in blocking solution for 2 h and then washed 5 times (20 min each) with the washing solution, PBST.

Subsequently, the membrane was incubated with the HRP labelled secondary antibody (Protein-L HRP, SIGMA) diluted to 1: 5000 in blocking solution for 1 h. Blot was washed 4-5 times (20 min each) with PBST and developed with DAB as substrate.

2.13 Recab expression at shake flask level

Shake flask cultivations were performed in 20 ml capacity Erlenmeyer flasks in BMMY induction medium and allowed to grow at 200 rpm.

2.13.1 Effect of methanol concentration on Recab expression

Methanol concentrations of 0.5, 1 and 1.5% (v/v) were used for theinduction at every 24 h. Recab yield and growth were monitored at every 12 h up to 108 h. The batch was performed at 30^0C. OD_{600} of the culture was measured and Recab expression level was checked by quantitative ELISA. The integrity of Recab protein was checked on 12% SDS-PAGE.

2.13.2 Effect of temperature on expression level

Three temperatures 25, 28 and 30^0C were used to check the expression and growth of Recab transformants. Transformants were grown in 0.5 % methanol induction media. Samples were collected at every 12 h up to 108 h OD_{600} of the culture transformants was measured at each sampling time. Recab expression was checked by quantitative ELISA taking different dilutions of purified recab as standard. 12% SDS-PAGE was used to check the integrity of Recab protein.

2.14 Estimation of anti TNFα scFv level using quantitative ELISA

The concentration of anti TNFα scFv in the recombinant *P. pastoris* culture medium was estimated using ELISA. Standard curve with different concentration of the purified protein was made; the level of expression was calculated according to the standard curve.

2.15 Total proteins estimation (Bradford's method)

Total protein in the protein extract and culture supernatant were determined using Bradford's dye binding method (Bradford, 1976).

2.16 Cell based assay

Confluent monolayer Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, penicillin, gentamycin and glutamine. 10^5 HeLa cells were seeded in 48 well cell culture plates. Cells were allowed to grow until monolayer was formed (18 h to 22 h). The medium was discarded after overnight culture and replaced by fresh DMEM media. Cells were washed with sterile Phosphate Buffer saline (PBS). hTNF α was added per well at a final concentration of 0.5ng/ml. In-house samples (yeast transformants) were added to the wells and plate was incubated at 37^0C for overnight. Purified Recab produced in *E. coli* was used as a positive control at a final concentration of 20µg/ml. IL-6 levels were measured by ELISA using commercially available kit. OD values were measured at 450 nm.

3. RESULTS

3.1 Expression of Recab in *Pichia pastoris*

3.1.1 Construction of a vector for Recab expression

Coding region of Recab (750bp) was amplified using ATNFF and ATNFR primers (**appendix Table 3**) from pITC2 phagmid. The PCR amplified Recab gene was digested and cloned at the *Cla*I and *Xba*I site of pPICZαC vector, in frame with α factor signal sequence. The resulting vector pPIC-Recab expression construct, was used further to generate vector with two copies of Recab named as Recab concatemer, according to the *in vitro* multimerization procedure described earlier (Brierley, 1998). In brief, pPIC-Recab expression construct was digested with *Bgl*II-*Bam*HI to released Recab cassette, which was further cloned at unique site at *Bam*HI of pPIC-Recab expression construct (**Figure 19**). The resulting vector, Recab concatemer had two copies of the Recab expression cassette in head to tail configuration and retained a unique *Bam*HI site at the end of the expression construct. Recab expression construct was digested with *Pme*I and linear vectors were used to transform *P. pastoris* strains X33 and GS115. Similarly Recab concatemer were transformed using electroporation. The resulted transformants had Mut^+ phenotype and grew efficiently on methanol containing medium. Transformants were selected on YPD medium containing zeocin at a final concentration of 100μg/ml and confirmed using PCR and Southern blot analysis.

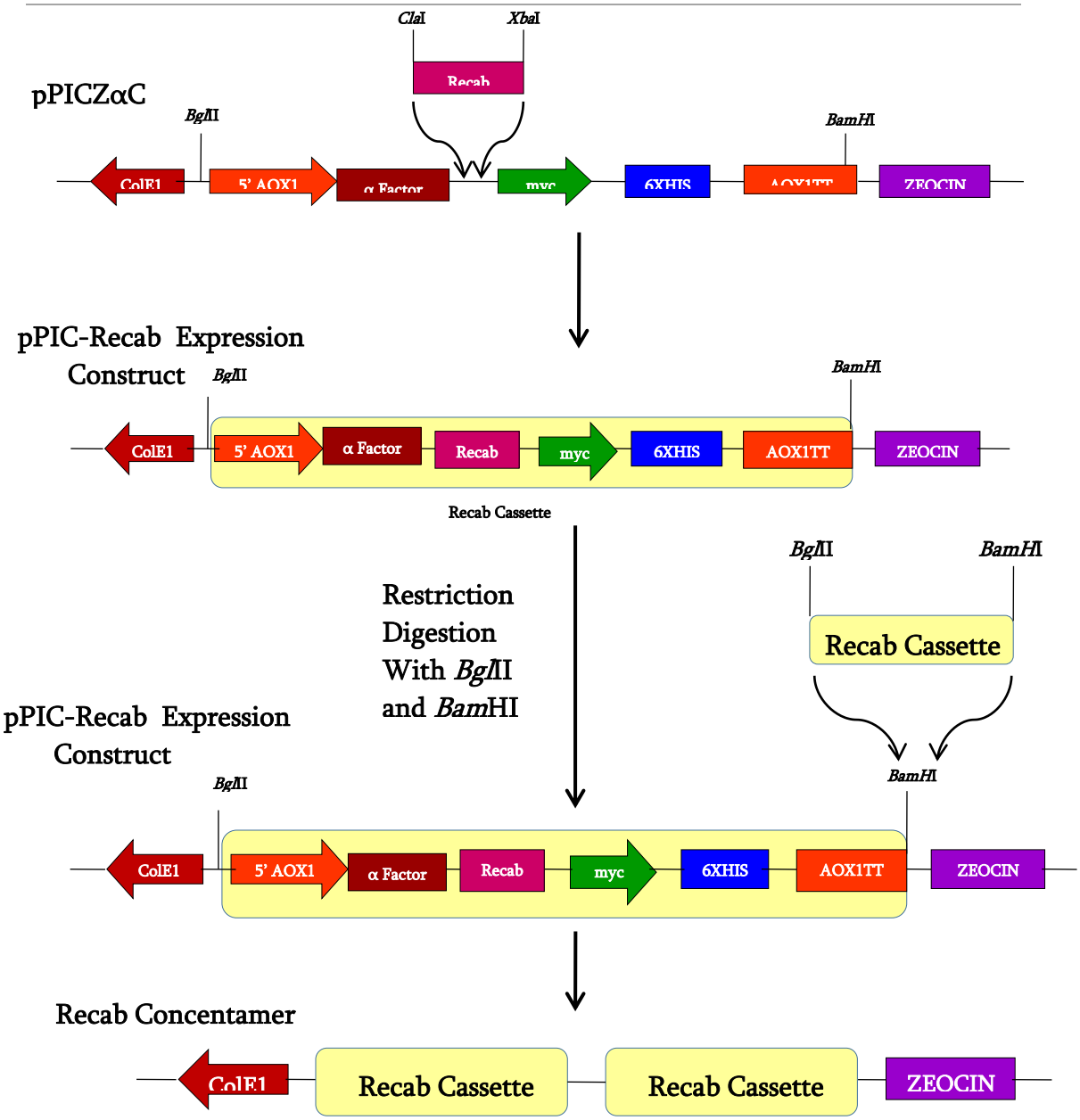

Figure 19: Construction of Recab expression construct for *P. pastoris*

Amplified Recab gene was digested with *Cla*I and *Xba*I and cloned at same sites of pPICZαC vector, resulted in pPIC-Recab expression construct. This construct was further digested with *Bgl*II and *Bam*HI to release Recab cassette, followed by cloning at *Bam*HI site of pPIC-Recab expression construct previously digested with *Bam*HI.

3.1.2 Confirmation of Recab recombinants of *P. pastoris*

P. pastoris transformants were analysed for the integration of expression cassette containing Recab gene into its genome. Colony PCR of the transformants was carried out using α factor F and c-myc R primers which resulted in a DNA fragment corresponding to the size of Recab (**Figure 20A**). The positive transformants were further confirmed using Southern blot hybridization. Genomic DNA of Recab transformants of X33 were digested with *Hind*III and hybridized with probe a (**Figure 20B**). Result showed that probe hybridized to three Recab concatemer (DC2, DC4 and DC5) at 2.3kb and ~4.5kb fragments indicating single integration while and transformants had single copy of Recab (**Figure 20C**). Similarly, genomic DNA of Recab transformants of GS115 strain were digested with *Hind*III and hybridized with probe a. It showed one transformant(Z9) of Recab concatemer with single integration showing 2.3kb and ~8kb band pattern and rest of the transformants had single copy of Recab (**Figure 20D**).

3.1.3 Analysis of Recab expression in *P. pastoris*

The expression of Recab in transformants was monitored by ELISA, RT-PCR, SDS-PAGE and western blot analysis. Supernatants were collected from the induced cultures after every 12 h of growth in methanol containing medium from 72, 84 and 96h time points and checked the expression level of Recab in transformants by ELISA. OD at 450 were read and plotted on a graph.

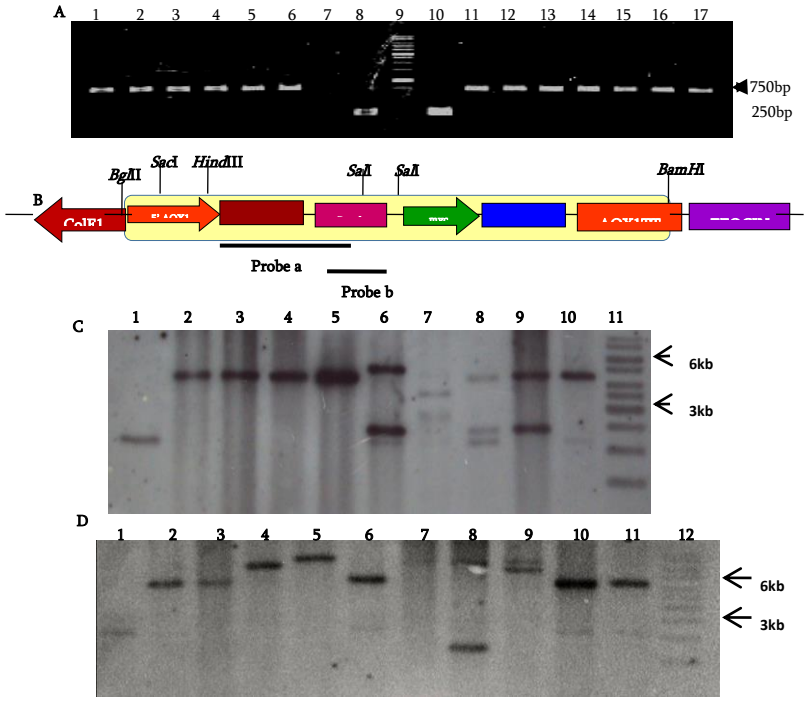

Figure 20: Screening and Southern blot analysis of Recab transformants

A. Screening of Recab transformants using colony PCR

Lane 1-6 and 11-17- Putative Recab transformants, Lane 7- Wild type strain, Lane 8 and 10- pPICZαC Control, Lane 6- DNA Marker.

B. Schematic representation of Recab expression construct showing different probes which were used for Southern analysis

C. Southern blot analysis of Recab transformants in X33 strain. **a**. Lane 1- X33 strain, Lane 2(CX2), 3(CX3), 4(CX4), 5 (CX5)- Recab transformants with single integration, Lane 6(DC2), 7(DC3), 8(DC4), 9(DC5), 10(DC6)-Recab concatemer transformants in X33, Lane 11- DNA Marker.

D. Southern blot analysis of Recab transformants in GS115 strain Genomic DNA of Recab transformants digested with *Hind*III and probed with **a**. Lane 1- GS115 strain, Lane 2 (Z1), 3(Z2), 4(Z4), 5 (Z6), 6(Z7), 7(Z8), 8(Z9), 9(CG6), 10(GC7) – Recab transformants, Lane 11- DNA Marker.

The result revealed that Z3, GC7, CX4, DC2 and DC6 showed higher OD values (higher Recab expression) compare to the other transformants (**Figure 21A**). Transformants with higher expression of Recab (based on the ELISA signal) were further analysed with Southern blot hybridization using *Sac*I and *Sal*I restriction enzymes. Genomic DNA of selected transformants was digested with *Sac*I and *Sal*I and hybridised with probe b (Recab) (**Figure 21B**). The result confirmed that DC2 was Recab concatemer transformant with single integration whereas rest had single copy transformants. The expression of Recab protein in these transformants was checked at transcript level using Reverse Transcriptase-PCR (**Figure 21C**) and a DNA band corresponding to 750 bp was obtained. The supernatants of 84h induced transformants were separated on 12% SDS-PAGE, a~28 kDa Recab protein was detected in all the transformants (**Figure 21D**). Confirmation of recab protein in supernatant was done by western blot analysis. Protein L-HRP was used for recab detection. A band corresponding to ~28 kDa was obtained in all the transformants. Representative single copy X33 (CX4), single copy GS115 (GC7) and double copy X33 (DC2) were selected for further analysis (**Figure 21E**).

3.2 Analysing effect of Temperature and Methanol concentration on Recab expression

Methanol concentration during the induction phase is a critical parameter and needs to be optimized with precision. Methanol concentrations of 0.5, 1, and 1.5 %

(v/v) were tested at 30^{0}C. Recab yield and the growth were monitored at every 12h up to 108h. As shown in **Figure 22** Recab yield was higher at a methanol concentration of 0.5% (v/v) in all transformants reaching to the maximum production level at different time points (GC7, 427 mg/L at 84h; CX4, 441mg/L from 36 h and 437mg/L at 60h). Growth of transformants was varying in different methanol concentrations as GC7 and CX4 showed high biomass at 1 and 1.5 % methanol concentration respectively (**Figure 22A2 and 22B2**) while DC2 grew slow at this methanol concentration, it grew better at 0.5 % methanol concentration (**Figure 22C2**). CX4 grew faster and biomass accumulation was very high compared to other two transformants, with an OD_{600} value of 29 at 108h whereas GC7 and DC2 grow very slow showing OD_{600} value of 14 and 10 respectively. Transformants were allowed to grow at different temperatures, 30, 28 and 25^{0}C. Recab expression did not show significant increase in X33 transformant, so to effect of temperature was checked on its expression levels. As lower temperature were reported to reduced protease activity in culture supernatant (Shi et al., 2003). Growth pattern and Recab expression levels were examined. There was no significant difference in growth rate of transformants at these temperatures. Recab yield was higher at 25^{0}C in all transformants compared to 30^{0}C. CX4 reached its highest expression level (500.9 mg/L) at 60h compared to GC7 (490.3mg/L) at 96 h and DC2 (501.8mg/L) at 84h (**Table 5**). Results indicated

that there was no correlation with extracellular Recab production versus copy number of Recab gene. No significant difference in level of Recab expression was observed when compared to single copy and double copy transformants.

Table 1: Quantitative analysis of expression of Recab protein at 25^{0}C in 0.5% methanol

ProteinYield (mg/L)	
CX4	501
GC7	490
DC2	501

3.3 Cell based assay for Recab protein obtained from *P. pastoris* transformants

The ability of the Recab to inhibit soluble hTNF α activity was examined using cell based assay. Hela cell lines were used to assay the effects of hTNFα, which induces expression and secretion of IL 6. Level of IL 6 can be determined by using ELISA. IL6 secretion is proportional to the concentration of the TNFα used for the induction. Functional activity of the Recabs was evaluated in Hela cell based assay by co-incubating with recombinant hTNFα (0.5 ng/ml). Purified Recab from *E.coli* was used as a positive control. Absorbance at 450 were noted down and plotted on a graph (**Figure 24**).Yeast expressing Recab inhibited expression and secretion of IL 6 comparable to the *E. coli* expressed. Activity of the recombinant proteins may vary from host to host, system from prokaryotic to eukaryotic. The results indicated that yeast expressed Recab neutralized hTNFα activity efficiently on HeLa cells.

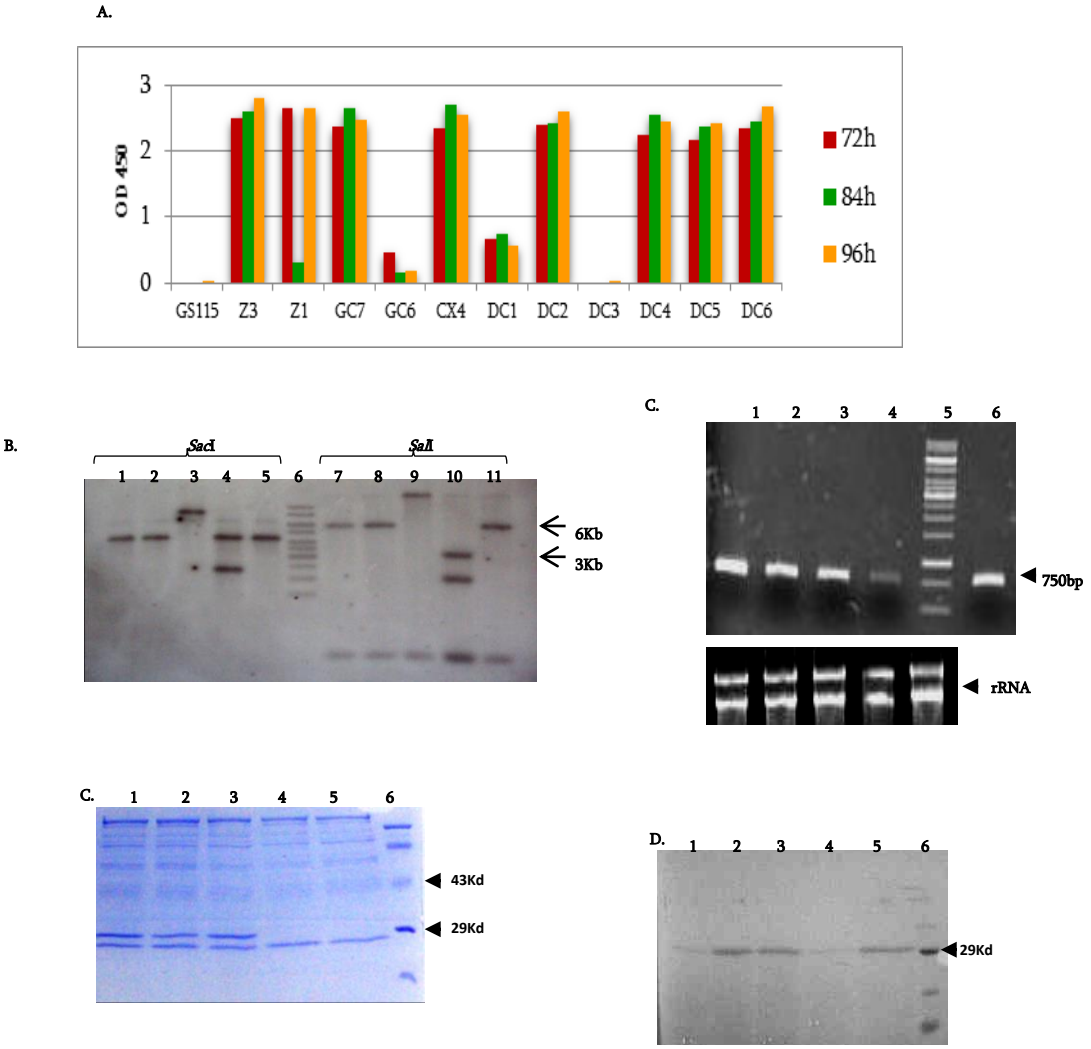

Figure 21: Selection of Recab transformants based on ELISA and Southern blot analysis

A. Recab transformants grown in induction medium for 72h, 84h and 96h at 30^{0}C and supernatant was used for Recab expression analysis using quantitative ELISA.

B. Genomic DNA of selected Recab transformants digested with *Sal*I and *Sac*I probed with Recab. Lane 1 and 7-GC7, 2 and 8- CX4, 3 and 9- Z3, 4 and 10- DC2, 5 and 11- DC6- Recab transformants, Lane 6- DNA Marker.

C. Expression analysis of Recab transformants with Reverse Transcriptase-PCR. Lane 1-Z3, Lane 2-GC7, Lane 3-DC2, Lane 4-DC6, Lane 5- DNA Marker, Lane 6-CX4. Lower panel: rRNA from corresponding RNA samples indicating equivalent loading of RNA.

D. Recab protein expression was checked on 12% SDS-PAGE. Lane 1- CX4, Lane 2- DC2, Lane3- DC6, Lane4-GC7, Lane 5- Z3 and Lane 6-Protein Marker.

E. Western blot of Recab transformants. Lane 1- Z3, Lane 2- CX4, Lane3- DC2, Lane4-DC6, Lane 5- GC7 and Lane 6-Protein Marker

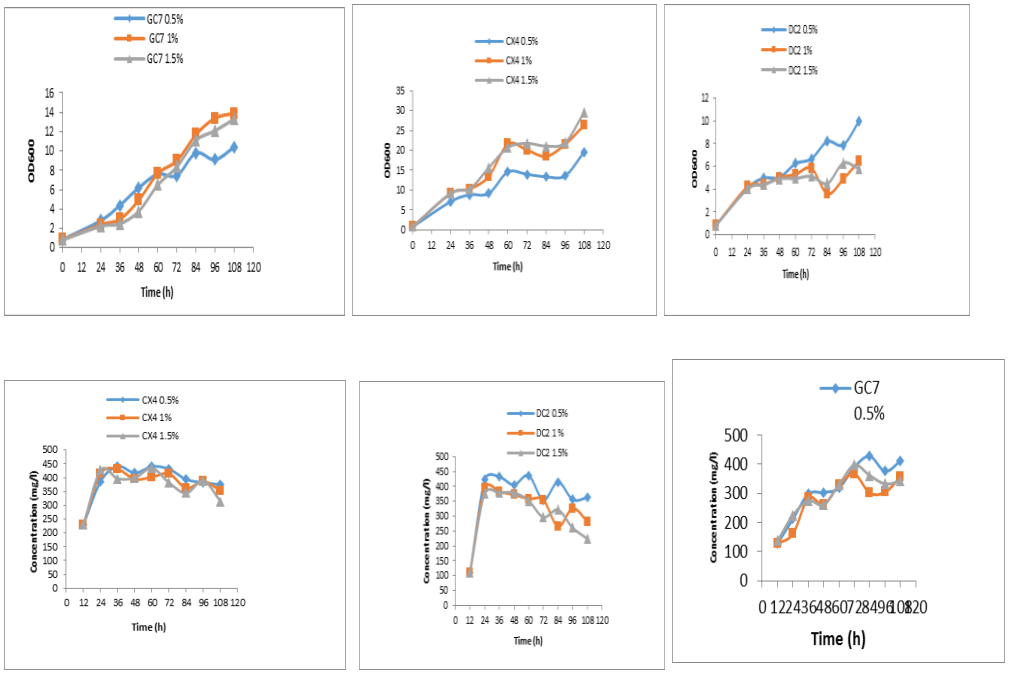

Figure 22: Effect of different methanol concentration on Recab expression Recab expression was checked in GC7, CX4 and DC2 and using 0.5, 1 and 1.5% methanol concentration at 30^{0}C up to 108h. Growth was monitored simultaneously.

A1. Effect of methanol concentration on protein expression in GC7 (Right panel). **A2.** Growth pattern of GC7 in methanol (Left panel).

B1. Effect of methanol conc. on protein expression in CX4 (Right panel).

B2. Growth pattern of CX4 in methanol (Left panel).

C1. Effect of methanol conc. on protein expression in DC2 (Right panel)

C2. Growth pattern of DC2 in methanol (Left panel).

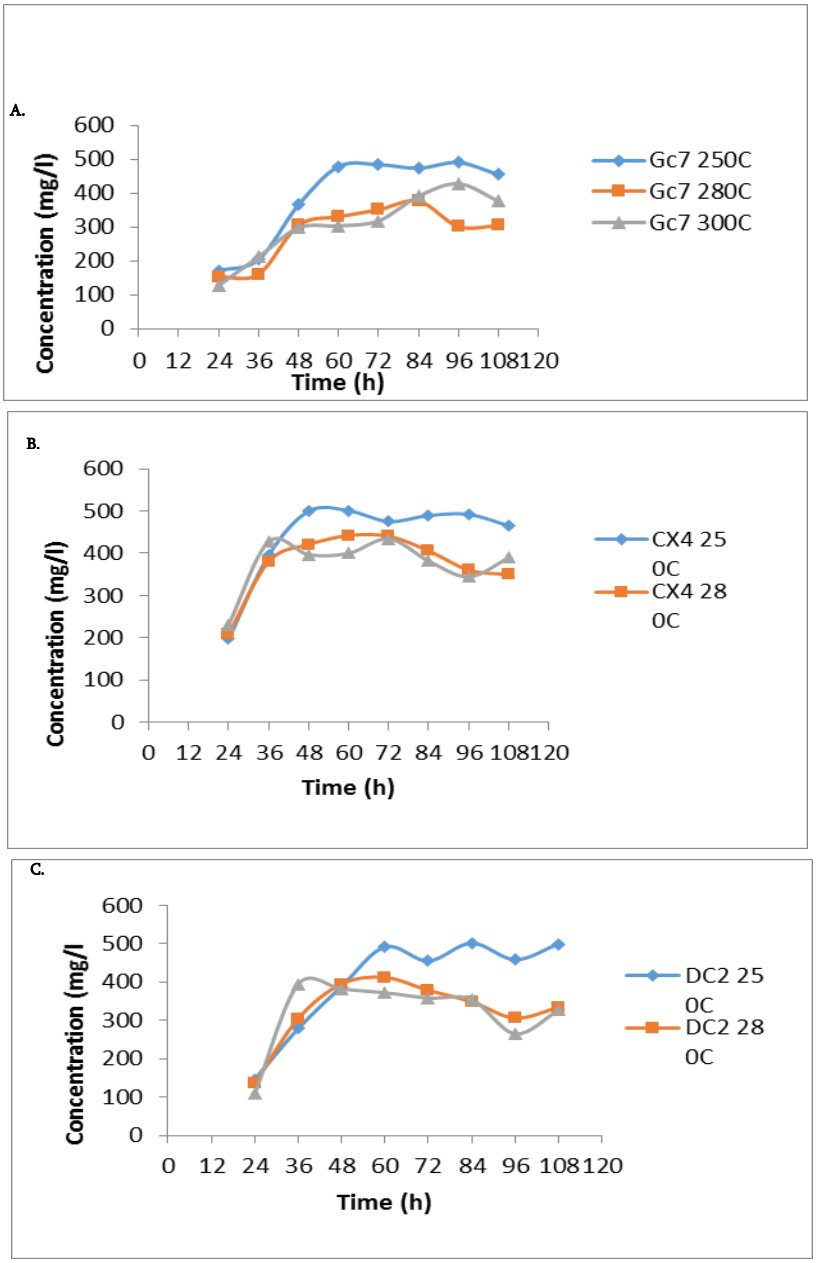

Figure 23: Effect of temperature on Recab expression Recab expression was checked in GC7, CX4 and DC2 at 25, 28 and 30^{0}C in 0.5% methanol concentration up to 108h.

A. Effect of temoerature on protein expression in GC7.

B. Effect of temoerature on protein expression in CX4.

C. Effect of temoerature on protein expression in DC2.

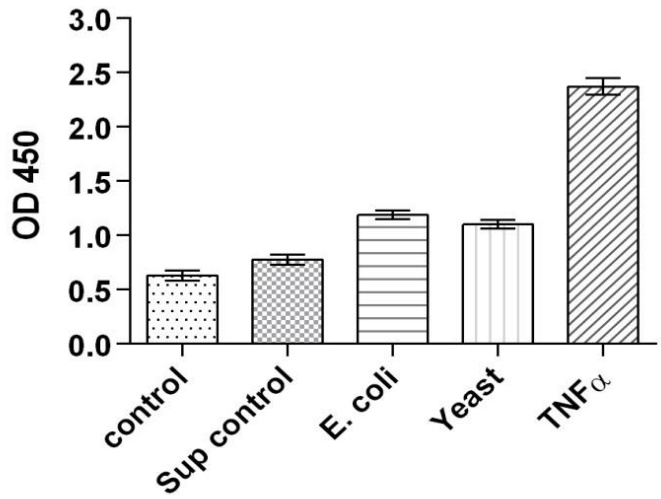

Figure 24: Cell based assay for Recab

Functional activity of Recab was evaluated in Hela cell line by co-incubating with recombinant human TNFα (0.5ng/ml). Purified Recab from *E.coli* was used as a positive control. Level of IL-6 was determined using ELISA.

Discussion

Successful expression of industrial enzymes and pharmaceutically relevant proteins has made *Pichia pastoris* one of the most widely used hosts for expression of heterologous proteins. It is rapidly gaining importance for the production of vaccines and pharmaceutically important proteins. In order to express anti-TNFα scFv (Recab), it was cloned in pPICZαC vector under the control of AOX1 promoter in fusion with α secretory sequence. X33 and GS115 strains were used for the expression studies. X33 being a wild type strain is better tolerant to higher methanol concentrations while GS115 is a well-studied strain for the herterologous protein expression. Expression vector containing single as well as double copies of Recab expression cassettes were constructed and transformed in *Pichia* strains. Transformants growing on zeocin were selected for further study. Integration of Recab in the genome was confirmed by Southern blot hybridization. Transformants were selected based on the copy number insertion and expression level of the Recab protein. Transformants with single copy insertion showing same band pattern in both strains were selected for further study. Expression of Recab was confirmed by ELISA and western blot analysis. Based on quantitative ELISA, concentration of the secreted Recab protein was estimated and compared among the transformants. Raemarkers et al., (1999), reported the inappropriate processing of α signal sequence of the secreted protein.

However, the present study, including SDS-PAGE and western blot analysis confirmed that α secretory signal sequence is properly cleaved and the mature Recab protein is secreted out in the medium. Increasing the copy number of expression vector in host strain was considered as a most useful strategy for increasing the expression level of the protein. Increasing copy number from 1 to 14 for tetanus fragment C protein (Clare et al., 1991) achieved 6 fold increases in expression levels. Similarly Werten et al., (1999) showed that increasing the copy number with secretory signal results in very high level expression of geletins (14.8 g/L). This observation was contradicted by Ahmad et al.,(2014) and concluded that there is no direct correlation between copy number and expression level. Present study also supports with the observations that there is no positive correlation between the copy number and expression level of Recab. DC2, a multicopy transformant in X33 strain, failed to show higher Recab expression level compared to CX4, a single copy transformant in X33 strain. Growth of DC2 was slow in comparison to CX4, it showed methanol sensitivity at higher concentrations. Similar results were reported by Cos et al. (2005), where they have shown that specific growth rate and methanol consumption capacity of high copy *P. pastoris* strain were abnormally reduced as compared to single-copy strain. This negative effect could be more pronounced in strains with higher gene dosage. Growth of CX4 was more than two fold higher compared to DC2 and GC7 (single copy in

GS115 strain) with an OD_{600} value of 29 at 108h in 1.5 % methanol followed by GC7 (OD_{600} value 14 in 1% methanol at 108h). DC2 was slow growing and sensitive to methanol concentration as it reached maximum OD_{600} value of 10 in 0.5 % methanol. CX4 was tolerant to methanol in induction media even at a concentration of 1.5 % while GC7 preferred only 1% methanol in induction medium.

For induction of Recab 0.5 % methanol was optimal. At this concentration, all strains showed maximum expression compared to the other concentration (1% and 1.5%). We found that the expression pattern of Recab differed in different transformants. CX4 reaching at a level of 441 mg/L after 36h of induction, GC7 at 84h reached its maximum level of 427 mg/L while DC2 was 367 mg/L at 60h. Maximum expression of GC7 reached at 84 h of induction and then it dropped down while X33 strain transformant CX4 and DC2 reached the maximum level at 36h and 60 h respectively. The levels of Recab were higher after 24h of induction in both CX4 and DC2 and there was no significant increase in expression level further. It could be because of presence of protease in the medium. Optimum temperature for growth and induction for *P. pastoris* is 28^0C to 30^0C. At this range protease may destroy the recombinant protein. Shi et al., (2003) reported that there is low protease activity at lower temperature of induction phase. Similarly Jafari et al., (2011) highlighted temperature as an important factor as lowering the

temperature to 11°C resulted in high yields of recombinant protein production. So we induced the cultures at three different temperatures 25, 28 and 30 ^{0}C. In the current study, yield of the Recab increased with decreasing the temperature, indicating that there could be protease activity in the culture supernatant of induced culture. Decrease in temperature gave Recab expression of 490.1 mg/L, 501 mg/L and 501mg/L for GC7, CX4 and DC2 respectively. These expression levels are comparable or better than the reported scFv expression levels; Eldin et al., 1996 (250mg/L), Jafari et al., 2011 (21.4mg/L), Shi et al., 2003, (25 mg/L), Chang et al., 2008 (328 mg/L), Damasceno et al., 2004 (4.3g/L). Further optimisation with different temperature and pH may lead to higher Recab production.

Functional assay for the expression of scFv was carried out on HeLa cell line. Change of the host could lead to inactivation or loss of the activity of these molecules. The results confirmed that Recab was functionally active and was binding to TNF α in the solution. Higher IL-6 levels in the sample with TNF α were reduced on addition of the Recab. Considering *E.coli* expressed Recab as a control, the inhibition of the TNFα binding by yeast expressed Recab was comparable to *E. coli* expressed Recab, which confirmed the functional activity of Recab protein which were expressed in prokaryotic and eukaryotic expression systems.