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SCREENING OF VARIOUS PROMOTERS FOR INCREASED PROTEIN EXPRESSION

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ABSTRACT

One of the essential elements in the in expression of recombinant proteins in *Eshecichia coli* is a promoter. It is a sequence of DNA that drives the expression of recombinant protein production. Along with the choice of promoter a number of factors such as protein parameters, induction temperature, inducer, induction conditions and protein solubility play a major role in deciding the yield of target protein. In the present study, we have evaluated the strength of three different promoters (*phoA*, *cstA* and pLpR) and compared it with one of the strong promoters known, T7. A vector with these three promoters were constructed for expression of FCTRX 1 – 15 tagged proinsulin gene. Protein expressed was isolated, solubilized and purified using chromatography. Yields of proinsulin obtained from different promoter driven constructs was compared and it was observed that the inclusion bodies (IB) yield obtained from temperature inducible pLpR system was comparable with T7 system and this can be further increased by optimizing the fermentation conditions.

Keywords: *E. coli: Escherichia coli*; IPTG: isopropyl β – D – 1 – thiogalactopyranoside; *cstA*: Carbon starvation promoter; *phoA*: Alkaline phosphatase; IB's: Inclusion bodies; HCP's: Host Cell Proteins

INTRODUCTION

Escherichia coli is the first host in the history of biotechnology to be used successfully to produce recombinant Insulin for therapeutic use by Eli Lily in 1982 **(Swartz, 2001)**. *E. coli* was one of the dominant hosts for production of recombinant proteins until mid-90s, after which yeast and mammalian expression system took over. In many cases like production of antibodies and other therapeutic proteins which require post translational modifications to attain their proper confirmation, eukaryotic systems are required. Both prokaryotic and eukaryotic expression systems have its own strengths and weakness. Major disadvantages observed in *E. coli* expression system is inclusion body formation and lack of post translational modification (like glycosylation) but advantages are plenty (**Sahadev *et al*., 2007; Demain *et al*., 2009**) (i) fast growth kinetics (doubling time is 20 mins) (ii) high cell density cultures (> 200g/L wet cell weight) (iii) rapid intracellular product accumulation by inclusion body formation (iv) easier methods for genetic manipulations. With all these advantages *E. coli* is considered as an ideal host for recombinant protein production (**Rosano *et al*., 2014; Tripathi, 2016; Lazano *et al*., 2021**). With the growing markets for recombinant protein production, several researchers are interested in fine tuning the expression levels by validating different promoters, terminators, increasing / decreasing copy number of plasmids and developing secretory expression systems. A variety of promoters have also been tried for expression of proteins to evaluate their strength and induction conditions. Of which, till date T7 promoter used in the pET system is very popular and is induced by IPTG (a synthetic non – hydrolyzable analog of Lactose). In this system, phage T7 RNA polymerase (T7 RNAP) encoding DNA sequence is placed in the bacterial genome under the transcriptional control of lacUV5 promoter (**Studier et al., 1986**). Usage of chemical inducers such as IPTG and antibiotics is expensive and toxic. Presence of these chemicals in product and effluents are toxic to environment. To overcome these problems, other constitutive or inducible systems (pH, temperature, salt etc.) or starvation systems (Phosphate, Carbon etc.) can be exploited (**Valdez-Cruz *et al*., 2010**).

Apart from T7 system, thermo regulated system is extensively used for production of many recombinant proteins and peptides. In this system, either one of the strong Leftward promoter (pL) or rightward promoter (pR) drives the expression of gene of interest and the other one drives the expression of mutant repressor λcI857 (λpL/pLcI857). This system is inducible by heat shock, during transcription pL / pR is controlled by temperature sensitive repressor cI857. It represses pL promoter at temperatures (28 - 30°C) and gets inactivated at higher temperatures (37 - 42°C (**Villaverde *et al*., 1993; Nataraj *et al*., 2020**). Few major disadvantages associated with this system are (i) less cell mass (due to growth @ suboptimal temperatures) (ii) loss of protein yield due to higher temperatures (degradation, proteolytic digestion, shorter half-life) (**Steen *et al*., 2010; Bokinsky *et al*., 2011**) (iii) dual stress is induced on the cells; one is due to heat shock and the other one due to over expression of recombinant proteins. Nevertheless, this system should be still considered for high value therapeutic protein production (**Derman *et al*., 1993; Menart *et al*., 2003; Stephanopoulos, 2007;**).

Another tightly regulated promoter often used for recombinant protein production in large scale is the *phoA* promoter. *E. coli* alkaline phosphatase (*phoA*) promoter and its signal sequence is studied extensively for secretory expression of proteins. A lot of work is carried out to establish methods for expression of proteins using *phoA* promoter in shake flask, but studies on scaling up to fermentation level is less observed. Transcription from this promoter is induced when the phosphate becomes depleted from the media (**Agbogbo *et al*., 2020; Gundinger *et al*., 2022**). Apart from *phoA*, there is another promoter which gets transcribed during carbon starvation; *cstA* (Carbon starvation promoter). Most of the times in natural environments bacteria will have to survive prolonged periods of starvation **(Botsford *et al*., 1992).** It is seen that cAMP (cyclic AMP) levels increases during carbon starvation leading to induction of carbon starvation proteins (cst genes) which in turn requires cAMP – CRP [cAMP – cAMP receptor protein] complex for induction.

As the demand for therapeutic proteins are increasing, high level expression of protein at low manufacturing cost can be achieved by selecting suitable promoter and host. In our earlier studies, we had identified a peptide sequence from thioredoxin gene which can act as a fusion tag, named FCTRX 1-15 for overexpression of difficult to express peptide like proinsulin. Briefly, FCTRX 1-15 peptide sequence was fused with insulin gene was synthesized in B-C-A manner. Wherein B is full length B chain and A is full length A chain from human insulin gene. C corresponds to C chain, however full length naturally occurring sequence was not considered in the study. Instead "KR" (Lysine, Arginine) was inserted between B and C chains. It is well known from the earlier data that these two amino acids are sufficient for proper folding of these two chains. The molecular weight of this fusion construct is 9.12 kDa. In continuation to that study, now we have made an attempt to compare the strength and efficiency of promoters for expressing FCTRX 1-15 tagged proinsulin complex and comparing the yields with IPTG inducible T7 promoter (**Nataraj *et al*., 2020**).

MATERIAL AND METHODS

Microorganisms and plasmids

E. coli DH5α was used for the maintenance of plasmids and *E. coli* BL21(DE3) was used as expression host. FCTRX 1-15 tagged proinsulin gene was cloned into pET41b (+) vector.

Media, Chemicals and other Reagents

Luria broth, Luria Bertani Agar, Tris buffer, and Glycine from Himedia. Isopropyl β – thiogalactopyranoside (IPTG), Sodium dodecyl sulphate, Acrylamide, bisacrylamide, Biotin, thiamine, Bromophenol blue, Potassium phosphate, Sodium phosphate, Sodium chloride, Ammonium chloride, Calcium chloride, Magnesium sulphate, Glucose, Sodium sulphate, MOPS and Kanamycin from MERCK. Coomassie brilliant blue G-250 from Loba chemie. The cell cultures were grown at 37°C and 180 rpm. The cell density was measured at 600 nm in spectrophotometer.

Cloning FCTRX 1-15 tagged proinsulin gene into pET41b (+) vector and expression analysis

Codon optimized proinsulin gene was cloned into pET 41b (+) vector and protein expression was evaluated in BL21(DE3) cells, by inducing with IPTG. Yield of proinsulin was found to be low. Hence a fusion tag strategy was applied, wherein initially Thioredoxin protein was used as fusion tag and then it was truncated from both N and C terminus to derive a 15 amino acids peptide tag FCTRX 1-15. This sequence was found to be an ideal fusion partner for overexpression of proinsulin gene under the control of T7 promoter (refer Table 1 for FCTRX 1 – 15 tagged proinsulin. FCTRX 1-15 amino acid sequence was fused at the N terminus of proinsulin with enterokinase cleavage site (DDDDK - for removal of fusion tag) and cloned into pET41b (+) vector. FCTRX 1-15 tagged proinsulin / pET41b (+) plasmid was transformed into BL21 (DE3) competent cells and small-scale expression studies were carried out for few clones, by inducing cells with 1 mM IPTG at 37°C for 15 hours and 180 rpm in LB media. Induced cells were normalized to 0.5 O. D_{600}, pelleted, re-suspended and solubilized using sample solubilizing buffer. 25µl of sample was analyzed on Tricine SDS PAGE. Protein bands were visualized after Coomassie staining. This construct was used as a control for comparing the strength of other promoters.

Table 1 Amino acid sequence of FCTRX (1-15) proinsulin

FCTRX (1-15) proinsulin amino acid sequence
MHHHHHHKVGALSKGQLKEFLDDDDKFVNQHLCGSHLVEALYLVCGERGFFYTPKTKRGIVEQCCTSICSLYQLENYCN*
Molecular Weight: 9.2 kDa Theoretical pI: 6.68

Cloning c*stA* promoter and expression analysis

cst A promoter system was constructed by modifying FCTRX 1-15 tagged proinsulin / pET41b (+) plasmid. Synthetic gene for *cstA* promoter corresponding to 176 bp was cloned in frame with FCTRX 1-15 tagged proinsulin at SphI and XbaI sites. The plasmid obtained was named as FCTRX 1-15 tagged proinsulin *cstA* / pET41b (+).

Small scale expression studies of c*stA* promoter

FCTRX 1-15 tagged proinsulin *cstA* / pET41b (+) plasmid was transformed into BL21(DE3) competent cells and transformants obtained were induced to assess the expression levels. Pre culture was initially grown in LB at 37° C and 180 rpm, then 1.0 O. D_{600} cells were transferred into 2L flask with M9 medium [consisting of Glucose – 0.2%, $MgSO_4$ – 1mM, $CaCl_2$ – 0.3 mM, Biotin – 1 µg, Thiamine – 1 µg, trace elements (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl) – 1X]. Cells were allowed to grow at 37°C and 180 rpm for 48 hrs to determine the time point at which CstA promoter gets activated. Cell death was observed due to starvation. To overcome this glucose concentrations in M9 media was reduced by half to attain complete depletion of glucose in lesser time, thus causing the cells to enter starvation phase. 0.5 O. D_{600} cells from was harvested at various time points such as 4 h, 8 h and 16 h along with cells grown in high concentration of glucose. Cells were washed with 10 mM Tris - HCl, pH 8 buffer to remove the traces of media. Cells were pelleted, re-suspended and solubilized using sample solubilizing gel buffer (62.5 mM Tris – HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β - mercaptoethanol). Samples were boiled at 100°C for 10 mins and 25 µl of sample was loaded onto Tricine SDS PAGE.

Cloning *phoA* promoter and expression analysis

phoA promoter system was also constructed by modifying FCTRX 1-15 tagged proinsulin / pET41b (+) plasmid. Synthetic gene for *phoA* promoter corresponding to ~450 bp was cloned in frame with FCTRX 1-15 tagged proinsulin at SphI and XbaI sites. Plasmid obtained was named as FCTRX 1-15 tagged proinsulin *phoA* / pET41b (+).

Small scale expression studies of *phoA* promoter

FCTRX 1-15 tagged proinsulin *phoA* / pET41b (+) plasmid was transformed into BL21(DE3) competent cells. Transformants obtained were induced by reducing phosphate concentration present in the media. Pre culture was grown in LB and then 1.0 O. D_{600} cells were transferred into LP (Low Phosphate) medium and XP (excess – phosphate) medium. Media composition used in the study was: 4.68 g/l NaCl, 1.5 g/l KCl, 1.08 g/l NH_4Cl, 0.35 g/l Na_2SO_4, 0.2 g/l $MgCl_2$, 29.0 mg/l $CaCl_2$, 0.5 mg/l $FeCl_3$, 0.27 mg/l $ZnCl_2$ and 12.0 g/l Tris base was sterilized after adjusting the pH 7.5 with HCl. 2.0 g/l Glucose, 20.0 mg/l each of L - proline and L – leucine, 1.0 g/l casamino acids were individually sterilized and added into the media prepared. 14.5 mg/l KH_2PO_4 was added to LP medium and 145.0 mg/L KH_2PO_4 was added to XP medium. Cells grown at 37°C, 180 rpm for 12 hours. 0.5 O. D cell samples were collected at 8 hrs and 12 hrs interval, lysed and analyzed on SDS Tricine PAGE (**Daum *et al.*, 1989**).

Construction of thermal regulated vector system

A thermal regulated vector system was constructed by modifying pET41b (+) vector. A synthetic cassette of pR promoter in frame with cI857 coding sequence was procured. This fragment was excised from pR-cI857/pMA plasmid using PvuII and MluI site and cloned into pET41b (+) plasmid at the same site. Colonies obtained were screened for the presence of pR – cI857 cassette and named it as pR/ pET41b (+). Another synthetic cassette with pL promoter in frame with FCTRX 1-15 tagged proinsulin gene was procured as pL - RBS - FCTRX 1-15 tagged proinsulin / pMA. From this pL – RBS – FCTRX 1-15 tagged proinsulin gene was excised using SphI and BamHI. This fragment was cloned into pR/ pET41b (+) and named as FCTRX (1-15) proinsulin pLpR/pET41b (+).

Small scale expression studies of thermal regulated promoter

FCTRX 1-15 tagged proinsulin pLpR/pET41b (+) plasmid was transformed into BL21(DE3) competent cells and small-scale expression studies were carried out for few clones. Initially cells were grown at 30° C with shaking until it reached an O. D_{600} of 0.8 to 1.0. During induction, cells were incubated at three different temperatures 35°C, 37°C and 42°C for 15 hours at 180 rpm in LB media. Induced and uninduced cells were normalized to 0.5 O. D_{600}, pelleted by centrifugation, re-suspended in PBS and solubilized using sample solubilizing buffer. 25 µl of sample was analysed on Tricine SDS PAGE followed by Coomassie blue staining.

Comparison of T7 promoter and pLpR promoter constructs in 2L fermenter scale

Fed Batch fermentation

FCTRX 1-15 tagged proinsulin clone under the control of pLpR promoter and T7 promoter was tested in 2L bioreactor for estimating the titers. Fermentation media used was similar to that any *E. coli* minimal media at large scale. 35 g/L yeast extract, 20 g/L DMH, 10 g/L KH_2PO_4, 9 g/L $MgSO_4$ along with 20 g/L of trace salts. Fed batch run is divided into growth phase and induction phase. pH at 6 and rpm at 1200 to 1300 was maintained. In growth phase cells were allowed to grow until biomass of 380 to 400 g/l was attained. Once the biomass reached desired levels the glucose feeding rate was maintained based on DO spikes to ensure glucose concentration is minimal and there is no excess accumulation. T7 clones attained biomass in ~ 8 hrs (37°C), whereas in pLpR clones (30°C) took 12 hrs as temperatures set for these two were different. During induction phase 1mM IPTG was added to the cells with T7 promoter system and temperature, rpm was reduced to 30°C and 800 respectively. Cells were maintained in this phase for 10 hrs and then harvested. With pLpR system induction phase was different. At every 4hr intervals temperature was increased from 30°C to 42°C for 2 hours and then maintained at 32°C until next heat shock. This cycle was followed for 16 hrs and the cells were harvested.

Isolation of inclusion bodies

Induced cells from both expression systems were harvested by centrifugation at 12,000 rpm for 10 mins. 2.5 g of cells was resuspended in lysis buffer (50 mM Tris + 5 mM EDTA + 150 mM NaCl) and lysed in high pressure homogenizer at 100 bar pressure. After lysis, IB's were harvested by centrifugation at 12,000 rpm for 25 mins. Weight of IB's obtained was noted to calculate the recovery after DSP.

IB's were solubilized as explained in our earlier work. Briefly, inclusion bodies were washed with wash buffer 1 and wash buffer 2 (**Nataraj *et al*., 2020**), and then 1 g of IB's was solubilized in 50 ml of buffer containing 80 mM Tris and 6 M urea, by constant stirring at room temperature. Solubilized protein was refolded by diluting with denaturing reagent using 80 mM Tris at 8 - 10°C and then cysteine of about 2 mM and cystine 4 mM was added to provide oxidizing condition for proper refolding by disulphide bond formation. Refolded sample was concentrated by ultrafiltration and separated from other contaminating proteins by His tag based affinity purification. Purified protein was further processed with enzymes such as enterokinase (for removal of fusion tag), Trypsin and Carboxypeptidase (for enzymatic conversion of proinsulin to Insulin) after citraconylation. Reaction was arrested by crystallizing the protein using 1% Zinc Chloride. Crystals were further processed through preparative RP – HPLC and finally analyzed in RP – HPLC column (**Hartley *et al*., 1998; Ferrer *et al*., 2003; Klose *et al*., 2004**).

RESULTS

FCTRX 1-15 tagged proinsulin gene cloned into pET41b (+) vector was transformed into BL21(DE3) cells. Of many transformants obtained, a few were randomly selected and grown at 37° C until it reaches an O. D_{600} of 0.7 – 0.8. Cells were induced with 1 mM IPTG and incubated at 37° C for 15 hours at 180 rpm. Post induction, cells were normalized to 0.5 O. $D_{600,}$ and samples were prepared for analysing the expression level on Tricine SDS PAGE. Gel stained with Coomassie blue showed a distinct band at ~ 10 kDa, corresponding to the size of FCTRX 1-15 tagged proinsulin (Figure 1). This study was basically performed to appraise the strength of various *E. coli* promoters and compare it with T7 promoter. The DNA sequence encoding FCTRX 1-15 tagged proinsulin, cloned into pET41b (+) vector was used as backbone plasmid for construction of plasmids with different promoters transcribing this gene.

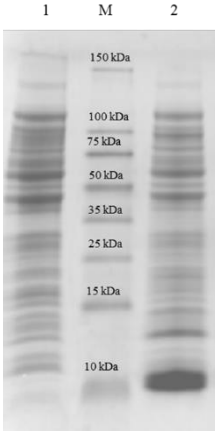

Figure 1 Expression of FCTRX 1-15 tagged proinsulin expression under the control of T7 promoter in *E. coli* BL21(DE3) cells induced with 1mM IPTG. Lane 1: Uninduced, Lane 2: FCTRX 1-15 tagged proinsulin Induced, Lane M: Broad range molecular weight marker Promega (V849A).

Synthetic *cstA* promoter gene was procured and cloned at SphI and XbaI sites by replacing T7 promoter. Plasmid obtained was verified by restriction profiling. FCTRX 1-15 tagged proinsulin *cstA*/ pET41b (+) plasmid, transformed into BL21(DE3) and one of the transformant was grown in LB media. Once the culture reached an O. D_{600} of 1.0, 1.0% inoculum was transferred into M9 media. Glucose concentration in M9 media was reduced by 50% to ensure complete depletion. Samples harvested at 8 h and 16 h intervals were analysed by electrophoresing on Tricine SDS PAGE. Samples showed a protein band corresponding to FCTRX 1-15 tagged proinsulin (Figure 2). This was further confirmed by western blot. Expression level was found to be less compared to T7 promoter and pL/pR promoter. This can be further improvised by optimization.

PhoA system was constructed similar to that of *cstA* system. Plasmid obtained was evaluated and transformed into BL21(DE3) cells. Transformants obtained were induced in low phosphate (LP) medium (**Studier *et al*., 1986**) containing 14.5 mg/l of KH_2PO_4 and excess phosphate medium with 145.0 mg/l of KH_2PO_4. Samples were collected at 8 and 12 h intervals. Cells were lysed using SSB and electrophoresed on Tricine PAGE (Figure 3). There was a significant amount of expression seen in samples collected from LP medium at 8 and 12 hours compared to cells from XP medium. A gradual increase in the expression level was observed with increase in time. This might be due to depletion of PO_4 during the course of induction.

Plasmid expressing FCTRX 1-15 tagged proinsulin with thermal inducible pLpR promoter system, was constructed by cloning a synthetic cassette with pR promoter controlling the expression of λcI857 repressor gene into pET41b (+) vector (Figure 4) at PvuII and MluI sites. To this plasmid, another cassette with pL promoter followed by RBS and FCTRX 1-15 tagged proinsulin gene was cloned at SphI and BamHI site by replacing T7 promoter and GST gene. Efficiency of pLpR promoter system was tested first by performing small scale expression studies. To avoid leaky expression cells were initially grown at 30°C, until it reached 1.0 O. D_{600}. Then, cells were induced by increasing the temperature to 35ºC, 37°C and 42°C. Induces samples were electrophoresed on the Tricine SDS PAGE and visualized after Coomassie staining (Figure 5). Protein band corresponding to FCTRX 1-15 tagged proinsulin was observed in all the temperatures. Based on the gel profile 37°C and 42°C inductions showed slightly higher level of expression compared to 35°C. But the fact that expression level with T7 promoter was found to be higher than thermal inducible system remained same.

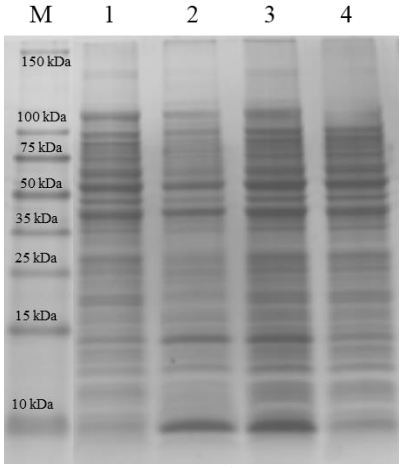

Figure 2 Small scale expression studies of proinsulin under the control of CstA promoter; Lane M: Broad range molecular weight marker (Promega; V849A); Lane 1: Uninduced control; Lane 2: Expression at 4 h; Lane 3: Expression at 8 h; Lane 4: Expression at 16 h.

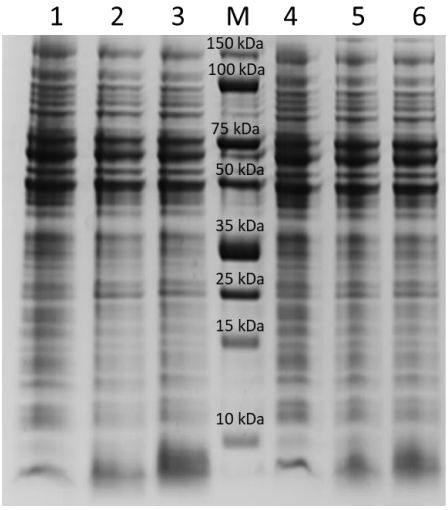

Figure 3 Small scale expression studies of proinsulin under the control of PhoA promoter; Lane M: Broad range molecular weight marker (Promega; V849A); Lane 1: Uninduced in XP medium; Lane 2: Expression in XP medium at 8 h; Lane 3: Expression in XP medium at 12 h; Lane 4: Uninduced in LP medium at 0 hr; Lane 5: Expression in LP medium at 8 h; Lane 4: Expression in LP medium at 12 h.

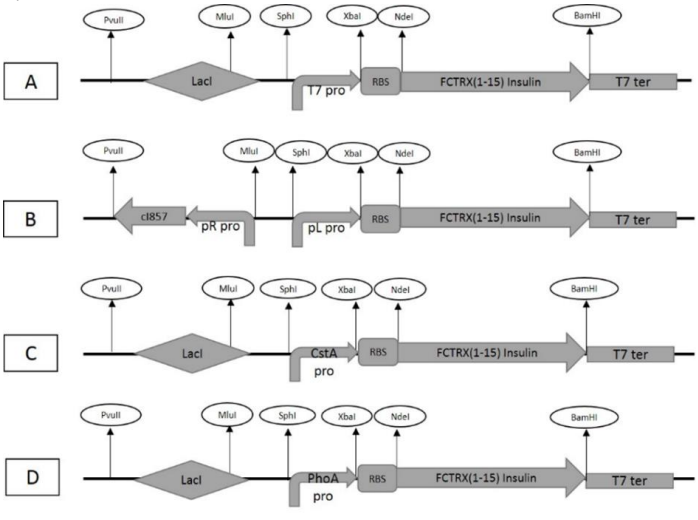

Figure 4 Schematic representation of vector modifications by replacing promoters

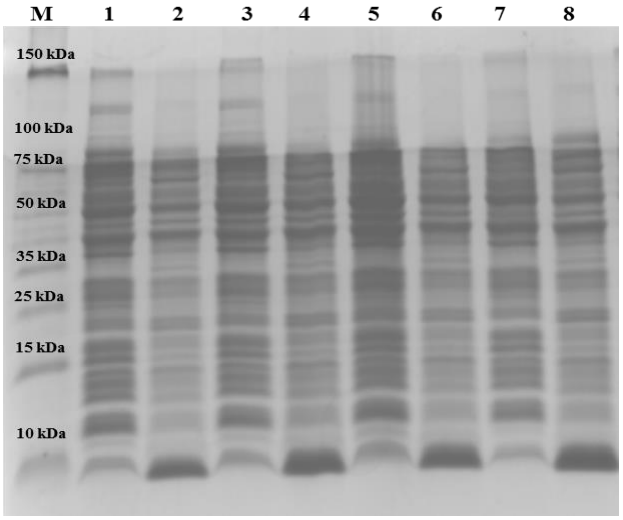

Figure 5 Small scale expression studies of proinsulin under the control of pLpR promoter; Lane M: Broad range molecular weight marker; Lane 1: Uninduced control at 35°C; Lane 2: Induced at 35°C; Lane 3: Induced control at 37°C; Lane 4: Induced at 37°C; Lane 5: Uninduced control at 42°C; Lane 6: Induced at 42°C; Lane 7: Uninduced T7 promoter control; Lane 8: Induced T7 promoter control.

From the fed batch fermentation of both T7 and pLpR construct cells harvested were subjected to lysis and Inclusion bodies were isolated. Approximately 1.0 g of IB was processed by washing with buffer containing Triton X 100 to remove cell related impurities and enhance the purity of IB's (Fig.6). After wash, IB's was solubilized using 6 M urea and total protein content was detected using BCA method. Total protein was found to be 300 mg/g of IB's (T7) and 234 mg/g (pLpR) repsectively. Affinity purification step was included to enrich the protein in the solution. His tag added to the N terminus of the construct was to assess the feasibility of using NiNTA column for purification. However, later we realized addition of affinity column step to the existing process will increase the CoGS and reduce the recovery. In the successive trials this step was excluded.

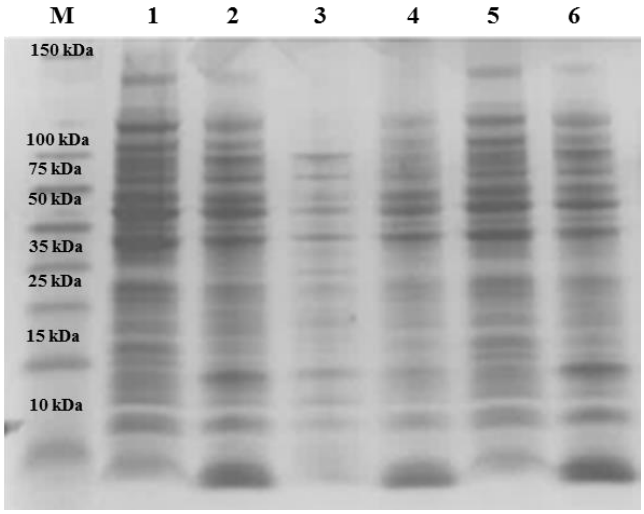

Figure 6 Large scale expression studies of FCTRX (1-15) proinsulin under the control of pLpR; Lane M: Broad range molecular weight marker; Lane 1: Uninduced T7 promoter clone; Lane 2: Induced with T7 promoter ; Lane 3: Uniduced pLpR system; Lane 4: Induced pLpR system; Lane 5: Uniduced pLpR system; Lane 6: Induced pLpR system

Solubilized protein was refolded using denaturing agents and finally digested with Enterokinase to release FCTRX 1-15 peptide tag from fusion protein. FCTRX 1-15 tag was separated from proinsulin using HEA chromatography. Proinsulin was citraconylated using citraconic anhydride, to mask free lysine and arginine residues from getting cleaved by Trypsin. After tryptic digestion, proinsulin was treated with Carboxypeptidase B, to remove lysine or arginine overhangs. Enzymatic reaction was arrested by crystallizing the protein using Zinc chloride. Crystals was dissolved in 2 N acetic acid with continuous stirring and finally purified using two rounds of RP – HPLC. Purified product was analyzed in RP – HPLC and compared with the standard Eli Lily Insulin. Retention time of purified insulin was found to be the same as standard insulin. Mass of purified insulin was analyzed by electrophoresing the sample along with Eli Lily insulin on a reducing PAGE. The gel profile of both the samples displayed the same pattern and was found to be corresponding to two bands (A and B chain) of insulin (Figure 7 and 8).

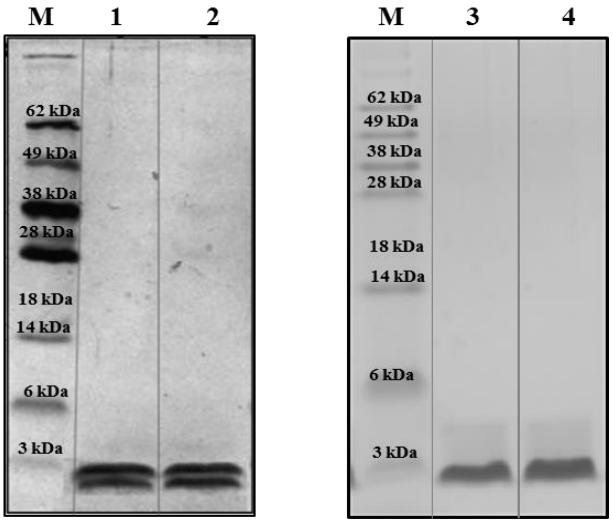

Figure 7 Tricine SDS PAGE profile of purified insulin from Thermal regulated system and Eli Lily insulin. Lane M: See Blue Prestained Ladder; Lane 1: Eli Lily Insulin under reducing condition, Lane 2: Purified insulin at reducing condition, Lane 3: Eli Lilly Insulin sample under non reducing condition and Lane 4: Purified insulin sample under non reducing condition

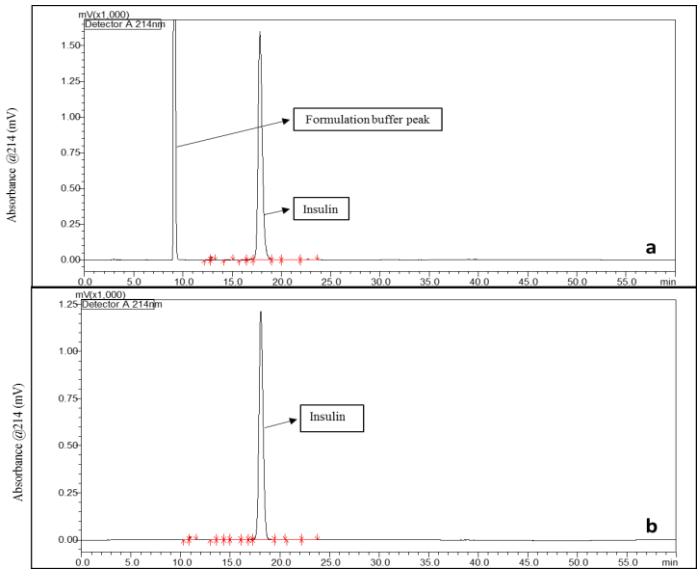

Figure 7 HPLC chromatograms for a. HPLC profile for Standard Eli Lilly sample; b. HPLC profile for crystallized insulin after preparative HPLC (Thermal regulated system).

DISCUSSION

We had previously described truncation of thioredoxin protein for generating a peptide tag which was found to be compatible with proinsulin and enhanced the expression of fusion protein several folds. Protein produced was further purified by developing a purification process and further compared with Eli Lily insulin. In this manuscript, we elaborate on developing an industrially viable clone with non - inducible / constitutive promoter for expression of proteins to reduce the cost of production. T7 promoter is one of the well-known strong inducible promoter which requires IPTG as inducer. The major drawback of T7 system is leaky expression and the cost of IPTG involved is too high. To overcome this, we shortlisted a series of promoters which can be induced by changing temperature either by increase / decrease in temperature and with other promoters chosen was supposed to get activated during the delpetion / starvation of active phosphate and carbon concentration. There have been many strategies employed to explore the possibilities of increasing the production of secondary metabolites and other industrially relevant enzymes by restricting the supply of required nutrients. There is also precedence in the literature about restricted supply of nutrients such as vitamins, nitrogen and phosphate limitation to enhance the yield (**Turner *et al.*, 1992; Dubey *et al.*, 2003**) Carbon / Glucose starvation is one of the areas to be explored extensively to understand the strength and efficiency of promoter *cstA.*

cstA promoter belongs to group of starvation inducible proteins and are known for their dependence of cAMP for post exponential phase induction. The promoter utilized herefor over expression of FCTRX 1-15 tagged proinsulin gene by reducing the glucose concentration in media. Further studies can be performed with this promoter construct by changing a few critical parameters during inductions as suggested by **Turner *et. al.*, 1992**. Continuous feeding of acetate to glucose starved culture arrests the growth and facilitates the continuous production of β – galactosidase and usage of glucose limited conditions in reactor promotes high cAMP levels, which in turn increases the productivity. This strategy can be tested to further understand the promoter efficiency (**Vasina *et al.*, 1997; Wilms *et al.*, 2001; Huber *et al.*, 2011**).

The next starvation / depletion-based promoter chosen was phoA promote. The inference which could be drawn from the experiment carried out with PhoA system is, it can be an alternative to T7, if certain parameters are optimized for large scale production such as determining the ideal concentration of PO_4 required for transcription initiation and process time required. During fermentation, there is always a requirement to achieve high biomass which will in turn lead to decrease in PO_4 concentration. But the presence of glucose in the media might help cells release cellular phosphate from hydrolysis (**Wang *et al*., 2005**). It was evident from the Tricine PAGE data, that the expression of FCTRX 1-15 tagged proinsulin increased constantly with increase in time due to the depletion of PO_4 present in the LP medium. But there was no significant increase in the r - protein production compared to the expression levels from either T7 promoter system or pLpR system. There are reports suggesting, at lower concentration of PO_4 (< 0.05 mM) cells starts expressing protein. A study from **Lubke *et.al.* 1995,** suggestes that "under PO_4 limitation, there was a significant loss of plasmid observed". Plasmid loss would in turn reduce the yield at later stages of fermentation. Considering all these facts, efficient feeding strategy has to be designed to effectively control both glucose and phosphate concentrations while maintaining the plasmid copy number. However, these two promoters (*cstA* and *phoA*) can be considered for expressing proteins which is of high value and low demand but might be challenging to consider them if high concentrations of recombinant proteins are required. Taking into all these facts, pLPR further optimization studies were carried out with pLpR system.

E. coli expression system is known to be the most convenient system for production of recombinant proteins. However, the challenge associated with this system is cost of inducer (IPTG) used during the process. Of the three promoter systems tried, pLpR system was found to show protein expression in par with T7 system. Currently, thermo induced expression system has gained interest among many researchers and has undergone many changes over a period of time. Different aspects such as over production of cI repressor gene, identification of lytic temperature sensitive mutants (cI857) and developing a temperature inducible T7 system etc. to stabilize this system has been tried by many researchers (**Restrepo – Pineda *et.al*., 2021**). Thermo regulated system constructed in this study is based on the insertion of a thermo labile repressor cI857 under the control of pR (rightward) promoter and FCTRX 1-15 tagged proinsulin gene under the control of leftward promoter. Gene expression is refrained at lower temperatures (between 28 - 32°C) due to the fine repression of promoter in the presence of cI857, whereas temperatures above 32°C initiates the transcription of host RNA polymerase and simultaneously inactivates the mutant repressor, thus ensuring the tight control of gene expression through temperature regulation. In T7, lac or trp promoter systems, the amount of repressor protein synthesized may not be sufficient, resulting in unsuccessful control and gene expression leakage. But in this thermo regulated system, there is a rigorous control of gene expression compared to other systems. As a consequence, our protein of interest is overexpressed without impacting the cell physiology and plasmid stability. Increase in temperature showed the presence of FCTRX 1-15 tagged proinsulin band. In other studies Expression level observed was found to be slightly lesser in cells induced at temperatures 37 and 42, when compared to 35°C, Even though the expression level is lesser in the thermal system compared to T7 system, the major advantage of this system is the reduction in the cost due to the absence of inducer such as IPTG.

As described by (**Lee *et.al*., 1988; Chao *et al*., 2004**) a two-stage process in bioreactor was performed with thermoinducible system to obtain optimal yield of recombinant proteins. In this process, the first bioreactor is maintained in repressed conditions i.e., at 37°C to accumulate biomass and preserve plasmid bearing cells, while the second bioreactor is operated at 40°C to initiate induction of recombinant protein production. In this arrangement, two interconnected bioreactors produced 30% more soluble β-galactosidase compared to batch culture, and the overall productivity and plasmid stability enhanced. After a careful assessment of this design, it was concluded that using an interconnected fermentor may not be a feasible option at large scale but this information led to design of a new bioprocess. For biomass generation lower temparatures were considered such as 32°C and then temperarture was increased. To attain the temperature precision during this fluctuation may be challenging at large scales, but at 2L scale it was found to be beneficial. As a comparison study, in the first fed batch experiment, temperature of 37°C was maintained constant throughout the growth phase as well as induction phase. In this experiment biomass did not increase beyond 100 g/L and expression level of protein was also optimum. This could be because of the initiation of protein expression from the initial growth phase which might be toxic to the cells. Continuously growing the cells at 42° C led to cell lysis hence a temperature shift process was found to be beneficial. But it was hard to increase and decrease the temperature in short span of time. Since there are no post translational modifications in *E. coli*, proteins expressed in this microenvironment leads to aggregation of proteins known as IB's. One of the major advantages of IB formation is increased yield of recombinant protein, but the disadvantage associated with the downstream is refolding of protein into its proper conformation. ~6.2 g of IB were obtained from T7 system compared to 4.0 g from 50 g of biomass. IB's obtained from the cells were isolated after cell lysis and was further processed to recover fully formed insulin (**Nataraj *et al*., 2020**).

For the protein to be biologically active, it must attain its three-dimensional conformation by formation of disulphide bonds. To achieve this in *in vitro* condition, IB's are solubilized in denaturing agents such as urea or guanidine hydrochloride and then refolded by diluting the denaturant and simultaneously providing redox reagents for oxidative sulfitolysis. This condition helps in proper folding of proteins. After refolding, fusion protein was subjected to enzymatic digestions for conversion of proinsulin into insulin. Followed by RP HPLC for purifying the mature insulin and removing closely generated species during enzyme digestions. Finally, insulin was crystallized using ZnCl. Quality of the protein crystals obtained was analyzed by HPLC and electrophoresing on Tris Tricine SDS PAGE. HPLC showed a single peak of > 80% purity corresponding to insulin, when compared with Eli Lily sample (**Hartley *et al*., 1998; Carrio *et al*., 2002; Nataraj *et al*., 2020)**. Tris Tricine SDS PAGE showed an intact molecule under reducing condition and in non-reducing conditions, there were two peptide bands corresponding to A and B chain. 80 mg of pure protein was obtained from 1g of IB from pLpR system compared 143 mg/ g from T7 system. IB yield can be further improvised by optimizing fermentation and induction parameters.

There are other well-known sugar inducible systems available in the literature such as Rhamnose, Arabinose and sucrose inducible systems. Feasibility of using these can be tested in future studies. However, the challenges associated in this system is apart from being an inducer these carbon sources gets consumed for meratbolic activity of the cells. Hence consumption of the carbon sources will be high leading to increased cost of production. To overcome this mixed feeding strategies can be tried in biorector (**Kosiba *et al*., 1982; Johnson *et al*., 1995; Khlebnikov *et al*., 2000; Giacalone *et al*., 2006; Wegerer *et al*., 2008; Li *et al*., 2013; Hjelm *et al*., 2017; Qin *et al*., 2021**) . Overall to summarise: Thermoinducible expression system can be one of the best alternative to T7 system for producing recombinant proteins. The bioprocess can be further fine-tuned to achieve: 1. Expression levels similar to that T7 system by inducing the thermoinducible system for production of recombinant proteins by fluctuating between high and low temperatures. 2. This system also allows precise control over the timing and level of protein expression, which can be adjusted by varying the temperature and duration of induction. 3. Major value add with thermoinducible systems are its cost-effectiveness because they do not require expensive inducers or complex regulatory mechanisms. 4. Its compatibility with fed-batch process; this can be further tweaked in thermoinducible systems as it maximizes the number of cells that can produce proteins. 5. The growth phase can also be extended to attain high cell densities in conjunction with sustaining the specific growth rate after thermoinduction. 6. Proteins produced using thermoinducible systems are often more soluble than those produced using other methods as the cells get comparatively more protein processing time, which can simplify downstream processing and purification **(Gupta *et al*., 1999; Aucoin *et al*., 2006; Caspeta *et al*., 2009).**

CONCLUSION

A successful expression system comprises of an ideal promoter which is tighly regulated, with efficient transcription rate but without causing any negative effects on the cell viability. Thus, it is important to choose the right promoter based on the objective to be achieved. Some promoters are regulated based on the presence (Arabinose, IPTG, Rhamnose etc.) and absence (Phosphate, Glucose starvation etc.) of compounds. But the ultimate goal is to choose a promoter which can yield upto 30% of the recombinnat protein from the total HCP's produced.

T7 system, induced by IPTG has time and again proved to be advantageous compared to all other systems in large scale. But the major limitation with respect to this system is increased cost of production at the large scale and plasmid instabilty due to physiological stress caused by the presence of IPTG.

To alaleviate these drawbacks, pLpR system with cI857 repressor techonology was incorporated in the study along with phoA, cstA starvation promoters. Insulin known to one of the most commonly used therapeutic drug, cost of production of this should be reduced to increase the affordability of of the drug. Based on the process ergonomics data, in the absence of IPTG the cost of production would reduce by 45% at large scale by maintaining the titers as in case of of IPTG induction. This could not be acheived with the starvation promoters conisdered in this study. However, pLPR system was promosing hence several strategies were tried in reactor with this system to achieve the objective but FCTRX 1 - 15 tagged proinsulin being a hydrophobic protein led to the formation of insoulble aggregates during continuous growth at higher temperaures leading to reduced recovery of expressed proteins. Hence a strategy was designed wherein, heat shocks were provided during the fermenttaion time, at regualar intervals to activate and deactivate the system. This led to increased production as well as recovery compared continuous growth at higher temperatures. Currently with pLpR system we observe 32 – 36% reduction in the cost but the target woulkd be reduce further

to match the requirement. To conclude, this strategy can be further optmized by developing a process based on the thermodynamic scheme during induction phase.

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