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Study on the Optimized Expression of *Pseudomonas Aeruginosa* Truncated Exotoxin A (PE38KDEL) in *Escherichia Coli*

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ABSTRACT: *The present study focused on the optimized expression of Pseudomonas aeruginosa truncated Exotoxin A (PE38KDEL) gene. This protein is considered as a potent toxin and is largely being used in the construction of immunotoxins for targeted cancer therapy. Escherichia coli BL21 DE3 Codon Plus was used as the expression host and the protein was expressed under the control of T7 promoter system. The effects of inducer type (IPTG and lactose), inducer concentration (IPTG: 0.2, 0.5 and 1 mM/lactose: 2.0, 5.0, 10, 15 and 20 mM) and incubation period (2, 4, 6, 8 and 10 hrs) were evaluated for the expressed protein. The maximum protein production was observed when IPTG at a concentration of 0.5 mM was used as an inducer however, lactose also showed substantial protein expression at 20 mM concentration. The most suitable incubation period for optimum protein expression was 6 hrs post induction.*

Keywords: *immunotoxins, promoter system, induction, lactose*

INTRODUCTION

Approximately four-fifth of dwellers on earth inhabits the less developed regions and hence 56% of newly diagnosed cancer cases and 64% of cancer deaths occurs in underdeveloped nations (Jemal et al., 2010). Consequently, cancer can be considered as a serious health risk for the people residing in Asian soils (Thun et al., 2010). It has turned out to be the primary death causal factor in Asian Pacific regions (Torre et al., 2015) and blamable for 3 million new cancer manifestations and 2 million mortal losses in Asia (Hanif et al., 2009). Pakistan ranks as the seventh most densely populated nation of the world. The political volatility, inflation and other uncertainties lead to increased incidence

of different diseases including cancer. In contrast to the past 10 years when cervical cancer was the chief death cause, now the breast cancer is responsible for utmost morbidity and mortality rates in women (Jemal et al., 2011).

New drugs to fight cancer have been and are being developed, novel therapeutic strategies are required owing to the higher frequency of drug resistance and toxic side effects resulting from the known treatments. The most challenging front in treating cancer for ages is the selective destruction of cancer cells leaving the normal body cells unharmed. Immunotherapy is a relatively new approach having the potential to combat cancer with the recently developed immunotoxins (FitzGerald et al., 2004). An immunotoxin entails an antibody joined to a toxin and is intended to explicitly exterminate tumor cells. The antibody component of the immunotoxin aims for antigens exclusive of cancer cells or profusely casing cancer cell surfaces and hypothetically spare normal body cells. The natural world harbors a vast diversity of toxins, encompassing lethal substances that are natural products of living organisms (Dosio et al., 2011). On average, only minute amount of toxin is required to mutilate cells, though the precise target and the toxic dosage may differ broadly. One of the novel therapeutic strategies being considered in hunt for promising cancer treatment relies on use of microorganisms namely live bacteria or their purified products. Microorganisms exude toxins as virulence factors in the course of pathogenic invasion or synthesize them as the products of secondary metabolism, infecting surrounding environments. Though normally unsafe and sporadically poisonous, various toxins can be exploited for therapeutic applications by modifications such as changing the method of delivery, altering the dose, reducing or synergizing particles (for instance from a heterogeneous mixture like venom). Their deadliness is heightened by the fact that these molecules hardly have any inhibitors. Diphtheria toxin (DT) secreted by *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa* exotoxin A (PE), and one of the virulence factors secreted by *Vibrio cholerae*, cholix toxin are prominent examples of such bacterial toxins and share many similar structural details. Changes in molecular structure may alter the hazardous properties of a toxin converting it into a beneficial therapeutic agent (Antignani and FitzGerald, 2013). A similar strategy has been applied in case of exotoxin type A (ETA) protein of bacterium *Pseudomonas aeruginosa. Pseudomonas* exotoxin a (PE) is amongst the most potent proteins secreted by its source bacteria (gram negative aerobic bacillus) which is an opportunistic human pathogen. The remedial value of this bacterial proteins has been established by their widespread used for the construction of recombinant immunotoxins (RITs) (Pastan et al., 2006, Pastan et al., 2007, Sarnovsky et al., 2010). The aim of this study was to

express the truncated and modified toxin protein of PE38KDEL that could be used for more potent immunotoxins.

MATERIALS AND METHODS

The previously designed recombinant plasmid PE38KDEL/pET22b was used in this study. The PE38KDEL gene was designed using overlap extension PCR technique and had already been cloned into the expression vector pET22b and the positive clones were confirmed using restriction digestion and sequence analysis. The plasmid was then transformed into *E. coli* BL21 DE3 Codon Plus using calcium chloride mediated transformation method.

Expression of recombinant protein PE38KDEL

Firstly, one or two of colonies of the *E. coli* BL21 DE3 Codon Plus with positive clones and empty expression vector were grown in 10 ml LB cultures containing the appropriate antibiotics at 37ºC overnight. Next morning, 200 µl of these cultures served as inoculum for another 10 ml cultures which were allowed to grow at 37ºC at 150-170 rpm until cells reached mid log phase (OD_{600} 0.6-0.7). At this point an aliquot from the culture with positive clones was stored on ice as a non-induced control. The remainder of the cultures were induced with 0.5 mM concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG). The incubation was continued at 37ºC with aeration for 6 hrs. At various time points during the incubation period (e.g., 2, 4 and 6 hrs and overnight), 1 ml of each culture was transferred to a microfuge tube. The cells in the tubes were harvested by centrifugation at maximum speed for 1 minute at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µl of 50 mM Tris-Cl buffer. After the addition of 1X Sodium Dodecyl Sulphate (SDS) gel loading buffer to the resuspended pellet, the samples were heated to 100ºC for 3 mins. The tubes were again centrifuged at maximum speed in a microfuge and the cell lysate was stored on ice until ready to load on a gel.

Optimization of the induction of target protein expression

The expression was optimized through small scale testing by analyzing the effects of various growth parameters such as; type of inducer (IPTG or Lactose), concentration of inducer and induction time. In these experiments, 10 ml of LB medium in 100 ml flasks were inoculated with freshly transformed *E. coli* DE3 colonies and the culture was placed in shaking incubator at 37ºC and 100 rpm. As the OD reached mid logarithmic phase, induction with various concentrations of IPTG (0.2 mM, 0.5mM and 1.0 mM) or lactose (2.0 mM, 5.0 mM, 10 .0 mM, 15.0 mM and 20.0 mM) for different time periods (0-10 hrs). While only one variable varied at a time with the others kept constant, 1 ml post induction samples were collected at different time intervals and bacterial growth was estimated using OD_{600}. Based on the

optical density, equivalent number of cells were pelleted, washed with 50 mM Tris-Cl buffered at pH 8.0, mixed with 1X reducing sample buffer, lysed at 100ºC for 3 mins and then finally run on 12% Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE).

The comparative distribution of expressed recombinant protein in soluble and insoluble cell fractions was also examined. The cells resuspended in 50 mM Tris-Cl were disrupted by sonication for one minute in ice box with 1-min intermittent cooling interval at 50 amplitudes (BANDELIN SONOPLUS HD2070) until the solution became translucent. Disrupted Cells were pelleted at 4ºC by centrifugation at 12000 rpm and supernatant was collected in a fresh tube. Both the supernatant representing the soluble fraction and the pellet were mixed separately with 1X SDS-PAGE Gel loading buffer, boiled for 3 mins at 100ºC and loaded onto 12% SDS-PAGE. Uninduced and induced control cell cultures were run in parallel for relative examination.

Analysis of expressed protein PE38KDEL on SDS-PAGE

The prepared samples were subjected to 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis to check the relative expression of the target protein. SDS-PAGE was carried out according to the method of Laemmli (1970). A protein gel casting system Bio-Rad was employed for the gel preparation using 8 x 10 cm glass plates. (Glass plates and combs were washed with 70% ethanol and deionized water and dried completely). The gel casting assembly was set up by placing the glass plates (separated by spacers) together and holding them with the aid of clamps. Once the gel casting system is established 15% resolving gel was formulated by mixing distilled water, 30 % acrylamide solution, 1.5 M Tris-Cl pH 8.8, 10 % SDS and 10 % ammonium per sulphate (APS) as mentioned in appendix. Tetramethylethylenediamine (TEMED) was added right before pouring the gel to initiate polymerization. The solution was swirled gently but thoroughly and appropriate amount of resolving gel was pippeted into the gap between the glass plates (leaving 1 cm vacant space at the top). To make the top surface of the resolving gel horizontal, water was filled into the gap until an overflow. Following polymerization (after 10-15 mins), 5 % stacking gel mixture was made and poured on the top of resolving gel (water discarded from above after it was gelated). Well forming comb was inserted carefully between the plates without trapping air under the teeth. The assembly was left undisturbed to polymerize at room temperature.

Once the polymerization was ensured, the comb was removed cautiously and the cassette containing the polymerized gel was placed into electrophoresis apparatus with upper and bottom reservoirs then filled with 1X Tris-Glycine buffer (wells washing was

performed in the same buffer). The prepared samples were warmed to the room temperature and 5 to 20 µl were loaded into the wells using fine tips. The gel was run at 60V for 60-80 min until the bromophenol dye reached the end of the stacking gel and then at 120V till the dye reached foot line of the glass plate.

After electrophoresis, the gel was taken out of the glass plates and immersed in Coomassie brilliant blue staining solution and slowly shaken on horizontal rotator for about 20-30 min. The gel was then transferred to the destaining solution and put on the same shaker for 2 to 3 hours until clear bands with almost no blue background were visible.

RESULTS

Protein Production

The recombinant production of foreign proteins in prokaryotic systems can be challenging and solubilization of desired protein may require a lot of time and effort. However, there is no uncertainty about the fact that production of recombinant proteins in microbial systems has transformed biochemistry. Primarily expression strains viz. *E. coli* BL21 Codon Plus (DE3) was the organism of choice for the production of recombinant protein (Fig. 1).

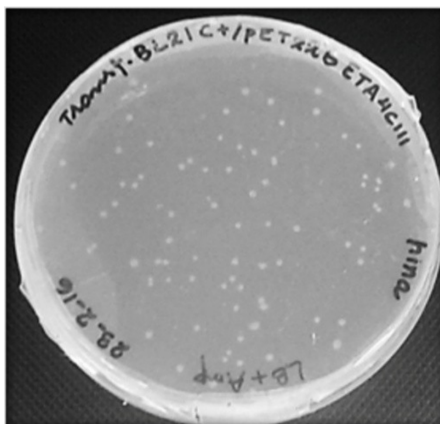

Fig. 1. *Escherichia coli* BL21 DE3 CodonPlus transformants cells on LB agar supplemented with ampicillin: White colonies observed are recombinant cells with plasmid vector PE38KDEL/pET

Expression of PE38KDEL protein in *E. coli*

E. coli BL21 Codon Plus (DE3) were transformed with PE38KDEL/pET, purified from *E. coli* DH5α and level of expression was investigated. Once the culture was grown to 0.6 OD, it was induced with IPTG at a concentration of 0.5mM and further allowed to grow for 6 hours post induction at 37 ºC. All the prepared control and induced cultures

were analyzed on 12% SDS-PAGE (Fig. 2).

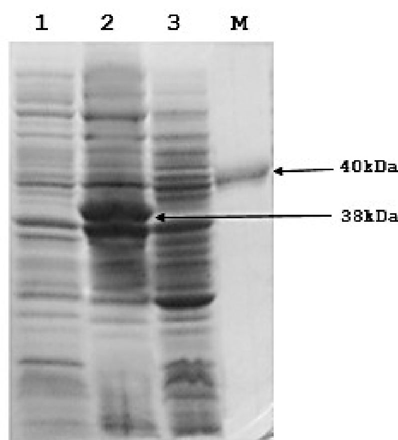

Fig. 2. 12% SDS-PAGE gel showing expression in *E. coli* BL21 DE3 (C+) harboring PE38KDEL/pET. Lane 1: Uninduced pET22b, Lane 2: PE38KDEL/pET induced (total cell lysate), Lane 3: empty pET22b (+) induced, Lane M: Protein Marker ovalbumin (40kDa)

Figure displays a notable difference in the profile of protein between the lane of bacteria with pET-22b (+) and the one with induced culture of bacteria containing pET22b/PE38KDEL. The presence of the expressed protein with the electrophoretic mobility of 38 kDa in Lane 4 confirmed that the recombinant protein was successfully produced.

Optimization of recombinant PE38KDEL/pET expression

The expression of PE38KDEL/pET was optimized using different concentrations of IPTG and lactose, post induction time and effect of host strain on expression.

Effect of IPTG concentrations on efficiency of induction

The concentrations of IPTG used to induce the lac repressor-regulated promoters can intensely effect protein expression. Keeping in view, IPTG at varying concentrations of 0.2 mM, 0.5 mM and 1 mM were tested from the suggested concentration range (0.01 mM-5.0 mM) to empirically optimize this parameter. A band of 38 kDa obtained on SDS-PAGE gel after Coomassie Blue staining R-250 in case of induced cultures confirmed the recombinant protein production. Analyzing the SDS-PAGE gel (Fig. 3), PE38KDEL expression was clearly visible at all the different concentrations of IPTG used after 6 hrs of post induction culture incubation.

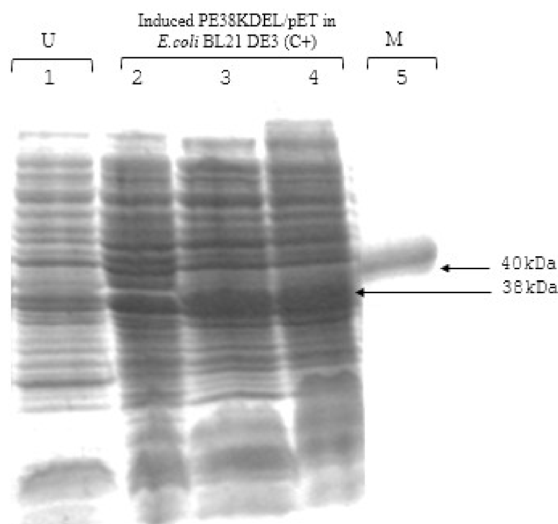

Fig. 3. 12% SDS-PAGE gel showing expression in *E. coli* BL21 DE3 (C+) harboring recombinant PE38KDEL/pET constructs at different IPTG concentrations. Host cells possessing PE38KDEL/pET constructs were induced using 0.2 mM, 0.5 mM and 1.0 mM IPTG at 37°C (0.6 OD) and cultured for 6 hours. The arrow labelled as 38 kDa indicates the predicted proteins (PE38KDEL). Lane description: Lane 1: 0.0mM IPTG, Lane 2: 0.2 mM IPTG, Lane 3: 0.5 mM IPTG, Lane 4: 1.0 mM IPTG, Lane 5: Protein marker ovalbumin (40 kDa)

*U= Uninduced, M= Protein marker

Maximum bacterial growth was obtained (OD_{600} 1.68) in culture induced at 0.2 mM IPTG after 6 hours in line for the less stress at this concentration on growth (Fig. 4). While the recombinant protein was being expressed in lesser quantity at 0.2 mM IPTG, the maximum protein production was observed in flask induced at 0.5 mM IPTG at 37ºC for 6 hours.

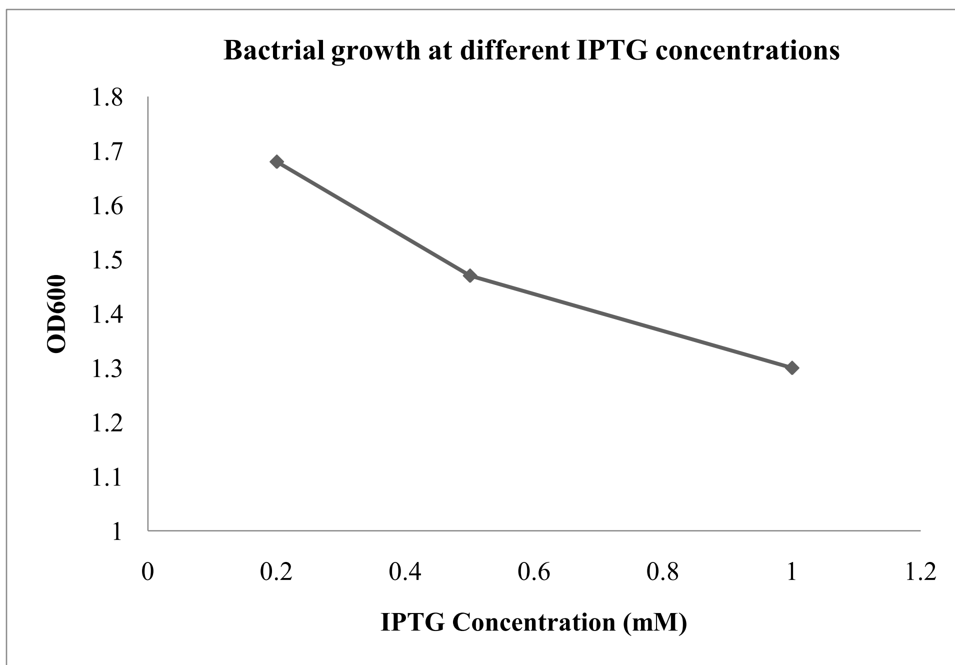

Fig. 4. The optical density of bacterial culture at different IPTG concentration after 6 hours post induction

Effect of different lactose concentration upon the induction efficiency

The expression of PE38KDEL/pET was further optimized by inducing the cultures of *E. coli* BL21 (DE3) CodonPlus with different concentrations of lactose. Fig. 5 showed the overall protein profiling of cultures induced at 2.0 mM, 5.0 mM, 10 mM, 15 mM and 20 mM lactose. Although the maximum growth was obtained in flask induced with 2.0 mM lactose, the best expression of PE38KDEL was observed in case of 20 mM lactose concentration.

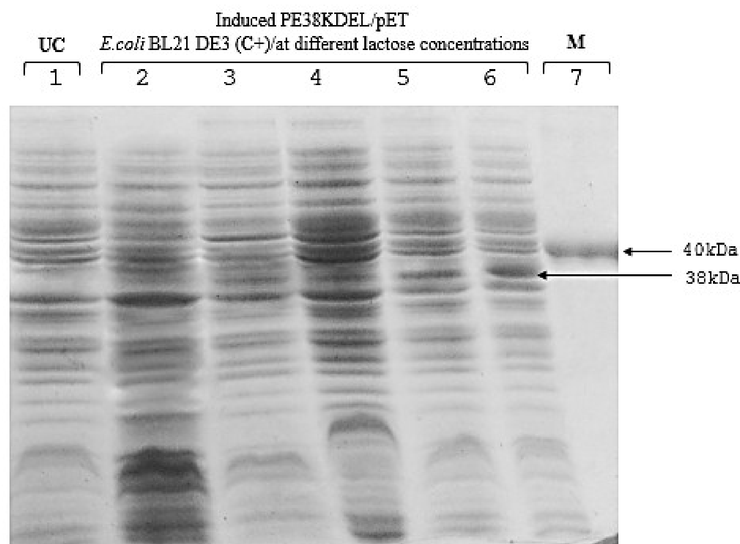

Fig. 5. 12% SDS-PAGE gel showing expression in *E. coli* BL21 DE3 (C+) harboring PE38KDEL/pET constructs at different Lactose concentrations. Host cells possessing PE38KDEL/pET constructs were induced using 2.0 mM, 5.0 mM, 10.0 mM, 15.0 mM and 20.0 mM lactose and cultured for six hours at 37°C (0.6 OD). The arrow labelled as 38 kDa indicates the predicted proteins (PE38KDEL). Lane description: Lane 1: 20 mM lactose (PE38KDEL/pET), Lane 2: 2.0 mM lactose, Lane 3: 5.0 mM lactose, Lane 4: 10.0 mM lactose, Lane 5: 15.0 mM lactose, Lane 6: 20.0 mM lactose, Lane 7: Protein Marker ovalbumin 40 kDa

The related values of optical density observed at different lactose concentrations is given in Fig. 6.

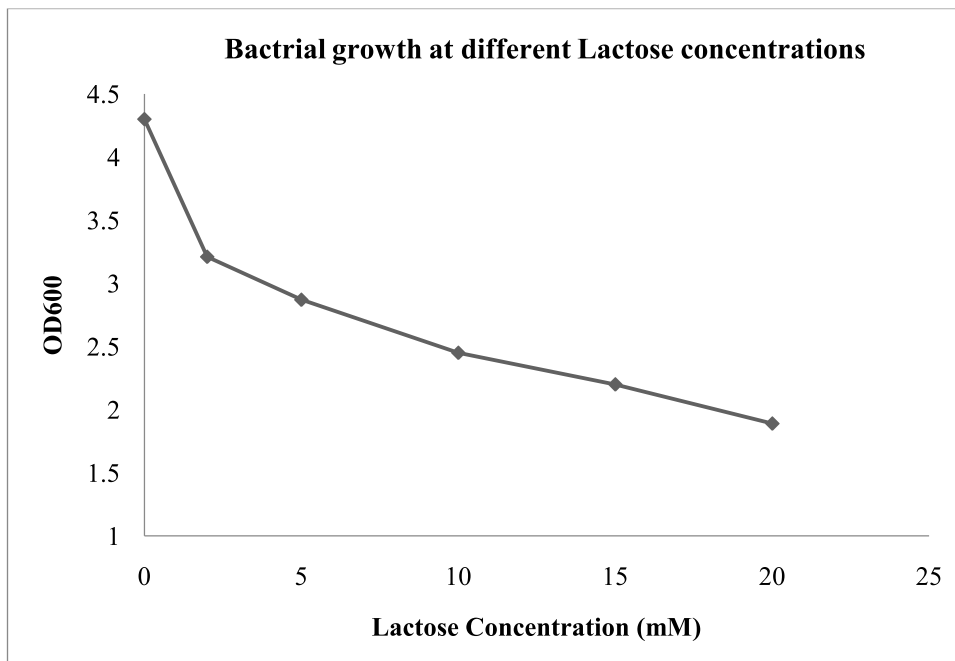

Fig. 6. The optical density of bacterial culture at different lactose concentration after six hours of culturing

Effect of varying incubation time post induction on PE38KDEL expression

To investigate the effect of growth period allowed post induction on production of PE38KDEL, the samples collected at different time intervals i.e., after 2, 4, 6, 8 and 10 hrs of cell culturing were screened for optimal expression of PE38KDEL. The protein expression was clearly visible at 2, 4, 6, 8 and 10 hrs after induction (Fig. 7).

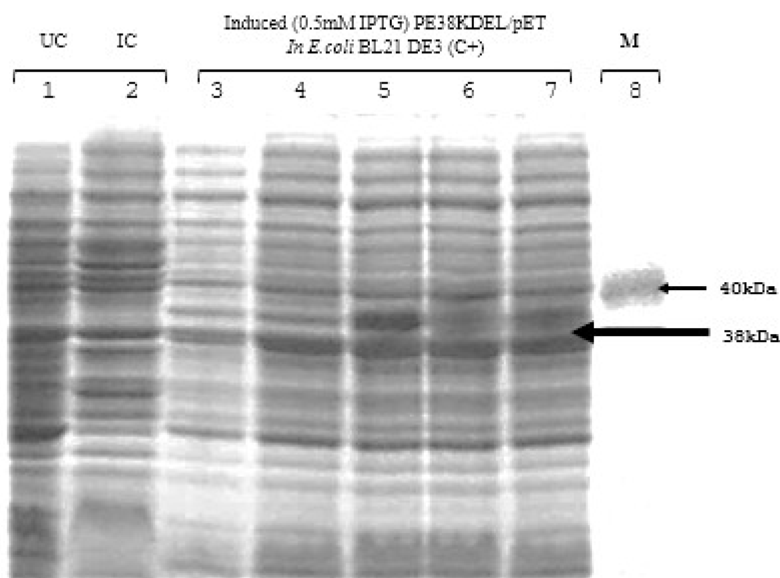

Fig. 7. 12% SDS-PAGE gel showing effect of time post induction in *E. coli* BL21 DE3 (C+) bacteria harboring PE38KDEL/pET constructs. Host cells possessing PE38KDEL/pET constructs were induced with 0.5mM IPTG at 37°C (0.6 OD). The arrow labelled as 38 kDa indicates the predicted fusion proteins (PE38KDEL). Lane description: Lane 1: 0.0 mM IPTG (PE38KDE/pET), Lane 2: 0.5 mM IPTG (pET22b), Lane 3 to 7: 0.5 mM IPTG (PE38KDEL/pET) t= 2hrs, 4hrs, 6hrs, 8 hrs, 10hrs respectively. 10-Protein marker ovalbumin (40 kDa)

*UC= Uninduced control, M= Protein marker

However, it was noted that the 38 kDa band was more pronounced in cell lysate sampled after 6 hrs of induction showing that maximum expression was recorded at this post induction time interval. The related values of optical density observed at different time intervals is given in Fig. 8.

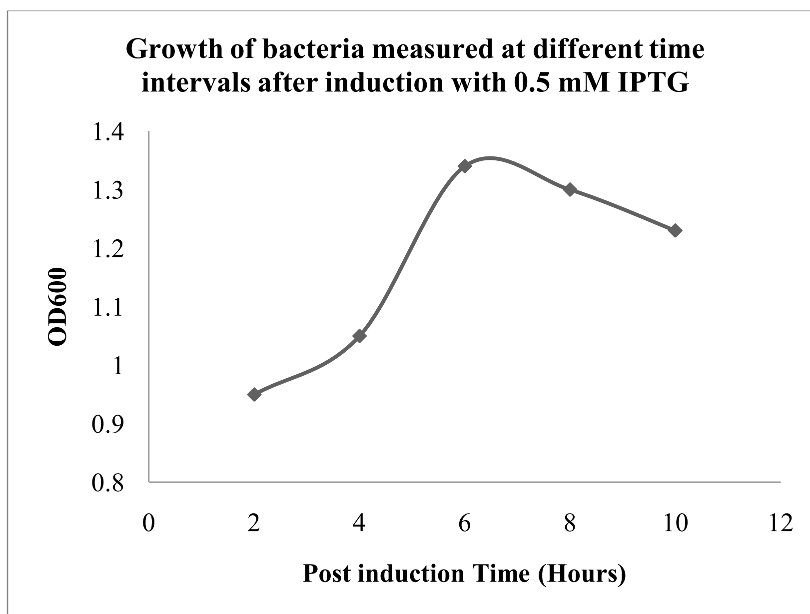

Fig. 8. The optical absorbance of bacterial culture at different time intervals post induction with 0.5 mM IPTG

DISCUSSION

Exotoxin A, produced as a virulent factor by many strains of *Pseudomonas aeruginosa,* is an extremely toxic protein having a median lethal dose (LD_{50}) of 200ng in mice (Engel, 2003). The protein expressed in this investigation was designed while considering the factors essential to cytotoxicity. The PE version produced encompassed 277-364 amino acids from domain II region and 395-613 amino acids of domain Ib and III. This PE protein was devoid of complete domain Ia and parts of domain II (253-276) and domain Ib (365-394). As described earlier in review of literature all these regions are inessential to the programming of toxin protein for immunotoxins. Moreover, as an example of AB toxins the cytotoxic domain A of PE remains stable and fully actively independent of receptor binding domain B. The resulting protein was termed PE38KDEL since it had 'KDEL' endoplasmic reticulum retention signal added at its carboxyl terminus. Two most important investigations have led to the exploitation of PE in immunotoxins construction designs (Allured et al., 1986, Hwang et al., 1987). The first one is related to the interpretation of the crystal structure of PE, revealing that the toxin molecule consisted of three major structural domains (Allured et al., 1986). The second investigation demonstrated that all three domains performed their functions as independent modules (Hwang et al., 1987).

For expressing the protein under the control of IPTG inducible T7

promoter using pET22b, suitable host organism was selected namely *E. coli* BL21 (DE3) CodonPlus and was grown in LB medium. This was selected for the reason that *E. coli* BL21 and its derivatives are by far the most successfully used host strains for expression studies. Standard growth medium viz., LB was used to establish the general parameters of the expression. Since foreign protein expression is affected by the rate of cell growth, factors such as the inoculum concentration in the growth medium, the incubation time for cell growth before induction and the density to which cells were cultured after induction were taken into consideration. Overgrowth or rapid growth leads to the overloading of the bacterial biosynthetic machinery and may cause formation of inclusion bodies. PE has previously been expressed in *E. coli* expression hosts using other expression systems such as pVC85, pET32a, pET28a and pET21a. (Baradaran et al., 2013, Kawooya et al., 1995, Kondo et al., 1988, Safari et al., 2014, Song et al., 2005). It was found out that the protein was being expressed in BL21 (DE3) CodonPlus when the culture medium was induced with the optimized 0.5 mM IPTG concentration as the optical density reached 0.6. However, the optimum lactose concentration for expression was found out to be 20 mM. The electrophoretic mobility of 38 kDa on SDS-PAGE showed the expression of recombinant protein. The analysis of effect of post induction incubation time revealed that high expression of PE38KDEL was obtained after 6 hours of culturing.

CONCLUSION

This study only focuses on the expression studies of PE38KDEL gene which has the potential to combat cancer cells. The expressed toxin protein could be used for designing more potent immunotoxins which come under the domains of targeted cancer therapy.

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