CHAPTER 6.

RESULTS & DISCUSSION

(Expression Studies in Shake Flask Culture)
Expression Studies in Shake Flask Culture

Since all the three clones obtained from the screening expressed scFvs with similar specificities with regard to binding with GM-CSF it was decided to study their expression, characterization so as to identify the clone which gave the best expression. It is well known that different proteins are expressed at different levels in E. coli and it is impossible to predict whether a protein will get over expressed or not. Thus these studies would help identify the most easily over expressible clone and that could be chosen for scale up studies if required. This property of ease of scale up and the ability to get large quantities of protein is indeed the major advantage of E. coli expression systems which is one of the primary reason for preferring scFvs over whole antibody production.

6.1 Effect of different media on intracellular (cytoplasmic) expression of anti GMCSF scFvs (Clone 4, Clone 196 and Clone VI)

The three expression vectors containing different scFv fragments viz. pET22b-scFv (4), pET22b-scFv (196) and pET22b-scFv (VI) were used to transform BL21 (DE3) cells. A single transformed colony from the plate was inoculated in 2 ml LB containing 1X (100 μg/ml) ampicillin and grown overnight at 37°C with continuous shaking at 220 rpm. 0.1 ml of this overnight grown primary culture was inoculated into 10 ml LB, 10 ml of LB modified and 10 ml of TB medium supplemented with ampicillin, for each clone and incubated under similar conditions. The objective was to observe the effect of different media on the recombinant protein expression transformed in BL 21 (DE3) cells. The optical density of the cells at 600 nm (OD_{600}) was monitored throughout the cultivation period. At an OD_{600} close to 1, cultures were induced with 1 mM IPTG and samples were collected at defined time intervals. 1 ml samples were drawn every hour post induction, centrifuged at 10,000 x ‘g’ and the cell pellets were stored at –20°C for analysis. The frozen cell pellets were resuspended in phosphate buffer saline (PBS) and 6X protein gel loading buffer was added to a final concentration of 1X, the
samples were boiled for 3-5 minutes. These samples consisting of total cellular protein were centrifuged at 12,000 x 'g' for 2 minutes to get rid of cell debris and the supernatants were loaded on a 12% denaturing and reducing polyacrylamide gel (SDS-PAGE). It was observed that the pET22b-scFv 4 clone expressed recombinant protein in decreasing levels in TB medium, LB medium and in LB modified medium respectively (Fig. 6.1).

Interestingly this clone gave almost comparable cell densities in LB, LB modified and TB, medium. The growing cultures (in all the three media) reached their maximum cell density within two hours of induction and then maintained almost the same cell density for the rest of the cultivation period for which we monitored their growth. thus the final cell densities were 2.4, 2.03 and 2.42 in LB, LB modified and TB medium respectively six hours post-induction (Fig.6.2).

However we observed that the build up of protein continued unabated inside the cell, even after stoppage of growth, to reach a fairly high level of a ~ 30% of TCP (Total cell protein) in TB medium. The second clone studied namely pET22b-scFv 196 showed the lowest recombinant protein expression levels in all the
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three media tested. It also expressed the recombinant protein maximally in TB whereas it was lower in LB medium. Almost no recombinant protein was seen in LB modified medium (Fig. 6.3). The SDS-PAGE pattern of scFv 196 recombinant protein shows two bands of almost equal intensity corresponding to the processed and unprocessed (pelB signal sequence) in LB and TB medium. This clone also showed poor cell growth in all three media tested. The cultures were induced at almost the same OD_{600} range of 0.9 - 1.4 and reached a final cell density of only 1.2, 1.9 and 2.0 in LB, LB modified and TB medium respectively six hours post-induction (Fig. 6.4). It is thus clear that post induction growth was negligible in all the three media. A possible explanation for this poor growth and also lower product yield could be that the processed protein (w/o the signal peptide) remains soluble and thus adversely affects the cellular health and also product formation. The pET22b-scFv VI clone also showed the lowest expression levels in LB modified medium as was observed with clone 196. As with the previous clones recombinant protein expression levels were higher and comparable in LB and TB medium. Here also two recombinant protein bands
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Fig. 6.3: pET22b-scFv 196 clone in BL21 (DE3) cells: SDS PAGE analysis of total cell protein of various hour of induction, a) in LB medium, b) in LB modified medium and c) TB medium

Fig. 6.4: Clone 196: Growth profile showing OD_{600} in different media.
corresponding to processed and un-processed (signal sequence) were seen in all the three media (Fig. 6.5). This clone again showed poor cell growth in all the three media tested. With LB and TB after slight increase in the cell density the 2nd hour post induction, cell densities started falling. However in LB modified medium the cell growth continued unabated after IPTG induction with negligible protein expression (Fig. 6.6). Clearly the build up of the protein inside the cell exerted a strong negative influence on cell growth. The expression levels were maximum in TB where after the 6th hour post induction the recombinant protein comprised approximately 40% of the total cellular protein.

Fig. 6.5: pET22b-scFv VI, clone in BL21 (DE3) cells: SDS-PAGE analysis of total cell protein of various hours of induction. A) in LB medium, b) in LB modified medium and c) TB medium
6.2 Effect of OD$_{600}$ of induction on expression of scFv proteins in TB medium.

From the previous expression studies for all the three scFv clones in different media (LB, LB modified and TB) it was clear that TB was the best medium for high level scFv expression. Therefore we decided to proceed with further expressions studies only in TB medium. The three clones were induced at early log phase and late log phase of growth in order to determine the effect of specific growth rate on expression and hence the optimum time point of induction.

For this all three pETscFv cells were grown in TB medium and induced with 1 mM IPTG at two different cell densities of OD$_{600} = 2.0$ (early log) and OD$_{600} = 6.0$ (late log) stage of growth. The cultures were grown for 13 hours post induction, 1ml samples were collected at different time points and were analyzed for scFv expression. The optical density of the cells at 600 nm (OD$_{600}$) was monitored.
throughout post induction period. It was observed that higher levels of recombinant scFv-4 expression were obtained in the cytoplasm in cultures induced at late log phase than in early induced cultures, which remained high throughout the cultivation period. Early induction of scFv-4 cultures resulted in low expression levels which fell further beyond the 4th hour post induction (Fig. 6.7).

![Graph showing expression studies in shake flask culture](image)

**Fig. 6.7:** pET22b-scFv 4 clone BL21 (DE3) cells in TB medium; SDS PAGE analysis of total cell protein of various hour of induction. a) induction done at OD600 of 2; b) induction done at OD600 of 6.

Induction of pETscFv-4 cells at early log phase resulted in a higher final cell density (OD\textsubscript{600} of 11.75), which was higher than even the un-induced control culture (OD\textsubscript{600} of 8.06) whereas induction at late log phase gave a final cell density (OD\textsubscript{600} of 8.5) comparable to the un-induced culture. The cells showed remarkably different growth patterns. IPTG induction at early log phase resulted in suppression of growth which was lost ~ 6 hours post-induction after which the
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cells grew fairly fast to reach cell densities higher than the un-induced control culture. On the other hand induction at the late log phase resulted in a gradual decline in growth rate which was similar to the decline observed with un-induced control. Thus this decline is more due to the starvation of nutrients in the culture rather than stress associated with protein expression (Fig. 6.8). However due to

leaky expression some protein was also expressed in the un-induced culture which is interestingly was higher than the final level observed with early log phase induction even though it was lower than late log phase induction. Thus protein expression does have a role in lowering the final biomass concentration which are achieved in the culture. Moreover the loss of expression coupled with a fast increase in biomass for the early log phase induction is possibly due to culture instability which allows non expressing cells to over take the population of expressing cells thus this experiment showed that late (OD\textsubscript{600} of 6) log phase

Fig. 6.8: pET22b-scFv 4 (BL 21) in TB medium: Growth profile showing OD\textsubscript{600} at a) induction with IPTG at early log phase, at OD\textsubscript{600}=2 and b) induction with IPTG at late log phase, at OD\textsubscript{600}=6
induction leads to higher levels of recombinant protein expression, possibly because it does not provide the culture with sufficient number of generations (cell doublings) for non recombinants to emerge in the cell population. During expression studies with the second clone namely scFv-196 cells, both induction at early log phase and late log phase resulted in low expression levels of recombinant protein. Early log phase induced cultures showed two processed and un-processed scFv protein bands, in SDS PAGE, of similar band intensity till 4th hour post induction after which protein expression fell significantly. Induction at late log phase also resulted in lower levels of expression of scFv196 protein and showed a single recombinant protein band corresponding to signal sequence processed protein on SDS-PAGE (Fig.6.9).

![Fig. 6.9: pET22b-scFv 196 clone BL21 (DE3) cells in TB medium; SDS PAGE analysis of total cell protein of various hour of induction. a) induction done at OD_{600} of 2; b) induction done at OD_{600} of 6.](image-url)
Induction at early log stage of pETscFv-196 cultures resulted in high final cell density $\text{OD}_{600}$ of 11.16, which was higher than the un-induced control culture ($\text{OD}_{600}$ of 7.71), and in suppression of growth which was lost ~ 4 hours post-induction. This loss of growth suppression relieved cells from metabolic burden as was evident from the significant low protein expression there after (SDS PAGE analysis). This pattern was similar to that observe with earlier clone. Induction at the late log phase also resulted in significant suppression of growth as there was negligible increase in cell density post-induction, which is indicated by the flattening out of the growth curve. However unlike the previous clone this suppression was lost ~ 6 hours post induction after which cell density increased rapidly leading to a final $\text{OD}_{600}$ of 10.13 (Fig 6.10).

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Fig. 6.10: pET22b-scFv 196 (BL 21) in TB medium: Growth profile showing $\text{OD}_{600}$ at a) induction with IPTG at early log phase, at $\text{OD}_{600}$-2 and b) induction with IPTG at late log phase, at $\text{OD}_{600}$-6
As with the previous clones, the expression of pETscFv-VI protein in TB was also higher when induced at late log phase as compared to the culture induced at early log phase of growth. Early log phase induction maintained reasonably high protein expression levels till the 4th hour post induction after that the expression level dropped drastically. This drop also coincided with the de-suppression of cell growth and here culture grew very fast to reach a final OD$_{600}$ of 11.4. This clearly demonstrates that the loss of expression ability correlates strongly with increased growth rates possibly reflecting the emergence of non recombinants in the culture medium. As opposed to this late log phase induction maintained higher protein expression levels till the 5th hour post induction and concomitantly very little growth was observed during this period (Fig. 6.11).

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**Fig. 6.11:** pET22b-scFv VI clone BL21 (DE3) cells in TB medium; SDS PAGE analysis of total cell protein of various hour of induction. a) induction done at OD600 of 2; b) induction done at OD600 of 6.
All the above results confirm that there is a strong relation between high level expression and growth suppression demonstrating the metabolic stress associated with recombinant protein expression. The major reason behind the decline in protein levels with early log phase induction is possibly the emergence of non producers which tend to grow faster since they do not carry the metabolic burden associated with scFv expression.

6.3 Localization of the recombinant single chain antibody protein inside the cell

The three clones were grown in TB medium and induced with 1.0 mM IPTG at an OD₆₀₀ of 2.0. 1 ml culture pellets were collected at the 4th hour post induction which represents the time point when maximum accumulation of the scFv s was observed. Sonication of these samples was performed at 4°C (30 seconds burst / 30 seconds cooling / 200-300 W) for 5 cycles. Lysed culture samples were then centrifuged at 12000 X g for 30 minutes at 4°C. Recombinant scFv proteins present in the supernatant comprised the soluble cytoplasmic fraction while the pellets, comprised the insoluble cytoplasmic fraction or inclusion bodies. All fractions were run on a 12% SDS-PAGE gel (Fig. 6.12). As is evident from the
Fig. 6.12: pET22b-scFv VI (BL 21) in TB medium: Growth profile showing OD_{600} at a) induction with IPTG at early log phase, at OD_{600}=2 and b) induction with IPTG at late log phase, at OD_{600}=6

gel the expressed recombinant scFv protein (for all the three scFv clones) was localized in the pellet and since double bands had been observed with two of the three clones indicating that the pelB signal sequence get partially processed, it was decided to look into the localization of the scFv inside the cell. Typically pelB cleavage indicated transport to the periplasm but this need to be confirmed, by preparing various cellular fractions and checking for the presence of scFvs. The supernatant did not show any recombinant band corresponding to the scFv protein (Fig.6.13).
Interestingly while the SDS-PAGE showed that the pET22b-scFv-4 clone was expressed as a single band. The pETscFv196 and pETscFv-VI clones showed two recombinant protein bands on SDS-PAGE corresponding to the processed and un-processed (signal sequence). To rule out the possibility of some contaminating protein which got pelleted along with the inclusion bodies, the pellets (post sonication) of all three clones were further given a 1% DCA wash to purify scFv inclusion bodies (see Materials and methods, 3.2.9). The purified inclusion body samples were again run on 12% SDS-PAGE gel (Fig. 6.14).
The SDS-PAGE of the purified inclusion bodies from the three scFv clones confirmed the observation that scFvs were being expressed in the form of inclusion bodies. While the pET22-scFv-4 clone was expressed purely as cytoplasmic inclusion bodies (single band). The pET22-scFv-196 and VI clones were processing the pelB signal sequence partially and thus giving rise to two bands. A possible explanation for this interesting observation is that the scFvs expressed from these clones do get translocated to the periplasm (along with the cleavage of the signal sequence). However due to a combination of high level expression and the intrinsic properties of the protein, they are unable to fold properly and hence form inclusion bodies in the periplasm (Hunke and Betton, 2003).