CHAPTER 4.

RESULTS & DISCUSSION

(SHAKE - FLASK LEVEL)
4 RESULTS

4.1 CLONING OF ASPARAGINASE GENE

The cloning of the \textit{E. coli} L-asparaginase gene (\textit{ansB}) was done by PCR amplifying it from a genomic DNA preparation of \textit{E. coli} K-12 strain (JM109). This involved the following steps.

\textbf{Isolation of genomic DNA:} An \textit{E. coli} JM109 culture was grown overnight, cells were harvested and genomic DNA was isolated by CTAB/NaCl method as described in material \& methods. The estimated yield of genomic DNA was 200 \(\mu\)g/ml with an \(A_{260}/A_{280} = 1.80\) suggesting a relatively pure DNA preparation free of contaminating protein.

\textbf{Primer design for PCR:} The nucleotide sequence of the asparaginase gene (\textit{ansB}) was obtained from GENE BANK (accession number M34234) and oligonucleotide primers were designed appropriately to incorporate restriction sites \textit{Nde I} and \textit{BamH II/Hind III} in the 5' and 3' end respectively (see material \& methods for primer details) to be used for directional cloning into various expression vectors.

Asparaginase being a naturally occurring periplasmic protein, the \textit{ansB} gene encodes a proprotein with a 22 amino acid N-terminal signal sequence and a 326 amino acid long mature protein. Primers were designed such that the starting 'AUG' codon was introduced through the \textit{Nde I} restriction site incorporated in the forward primer(s). 100 ng of the genomic DNA was used to set-up the PCR reaction to amplify the gene coding for Asparaginase using these gene specific primers (for PCR conditions refer M&M; Table 3.3)
The complete coding region of the asparaginase protein; \textit{ansB (+)} gene, and the coding region for the mature asparaginase protein; \textit{ansB (-)} gene, were amplified and cloned separately to generate a series of recombinant plasmid constructs (their properties in concise, are described in materials \\& methods; and construction details follow later in this chapter).

To facilitate the export of recombinant asparaginase, three different signal sequences were fused to the mature region of asparaginase protein a) asparaginase native signal sequence (described above), b) pelB signal sequence of pectate lyase B from another gram negative enteric bacteria \textit{Erwinia carotovora} - pelB was derived from pET-22b expression vector, c) Bacillus

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**Fig 4.1** Schematic illustration of PCR amplification of asparaginase gene with and without native signal sequence.

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**Fig 4.2** Schematic illustration of PCR amplification of Bacillus endoxylanase signal sequence.
endoxylanase signal sequence derived from gene coding for endoxylanase from Bacillus lyticus. The 84 bp *Bacillus* endoxylanase signal sequence (28 amino acid residues) was amplified from the recombinant plasmid pUC-XYL (Srivastava and Mukherjee 2001) using primers P5 (forward) and P6 (reverse) containing the *Nde I* and *Nco I* sites respectively (Fig 4.2).

### 4.2 CONSTRUCTION OF VARIOUS RECOMBINANT PLASMIDS CONTAINING THE ASPARAGINASE GENE

Two fundamentally different strategies for getting overexpression were planned and the cloning of the asparaginase gene was done accordingly. In the first strategy it was decided to express the asparaginase intracellularly hence the gene region coding for the mature asparaginase protein \(\text{ansB} \ (-)\) without any signal sequence was cloned directly under a strong T7 promoter with and without a histidine tag. The 6 x His tag was introduced at the N-terminus essentially to aid purification. In the second strategy, soluble asparaginase expression into the periplasm and/or in the extracellular space was attempted. For this the \(\text{ansB} \ (-)\) gene was cloned downstream of various signal peptides including its own native signal sequence in order to check the relative efficacy of secretion.

A number of recombinant expression plasmids were constructed in order to achieve high-level intracellular and secretory expression of asparaginase. PCR amplified asparaginase gene fragment(s) were cloned into series of expression vectors with varying characteristics (Table 3.2 in materials & methods). The generalized approach followed for generating of these recombinants was to double digest 1 µg of the expression vector by adding 1U each of the required restriction endonuclease and incubating the reaction mixture for 2 hours at 37°C. The PCR amplified DNA fragment was also double digested under similar conditions. Both the digested DNA fragments were run on TAE agarose gel (1% for the cut vector DNA and PCR amplified asparaginase fragments; and 2% for the PCR amplified endoxylanase signal sequence). The digested bands were excised from the gel and eluted using a gel extraction kit (Novagen, USA). Eluted
fragments were quantified spectrophotometrically by measuring absorbance at 260 nm. Ligation reaction was set up using a molar ratio of vector : insert :: 1 : 6 in 10 μl volume at 22°C for 16 hours. 1U of T4 DNA ligase was used per reaction. The ligase mix was heat inactivated at 65°C for 20 minutes and then used to transform 200 μl of freshly prepared competent E. coli DH5α cells. The entire transformation mixture was plated on LB agar petriplates containing 1X ampicillin (100μg/ml) or kanamycin (50 μg/ml), and incubated overnight at 37°C. From the colonies which appeared after overnight incubation a few colonies were picked up and inoculated in 10 ml LB medium containing 1X ampicillin and/or kanamycin and grown for 16 hours. Plasmids were isolated from these cultures and resolved on a 1% agarose gel along with control plasmids (the vectors without insert). All plasmids showing retardation in mobility compared to the control were selected for further screening. These plasmids were double digested with the same restriction enzymes used for cloning and the digested samples were run on 1% agarose gel to check for fall out of the insert of the desired size. The positive clones i.e. plasmids containing the inserts of the desired size were sequenced to confirm the correctness of the gene sequence.

Figure 4.3 A – 4.11 A show the schematic presentation of the construction of various expression vectors and figure 4.3 B to 4.11 B show the agarose gel pictures of the confirmations of the above clones by restriction digestion.
PCR amplified *ansB (+) gene

**Nde I** - **BamH I** digestion

**Ligation**

**Nde I** - **BamH I** digestion

Fig 4.3A - Schematic representation showing the construction of recombinant plasmid pT7Asp.
Fig 4.3B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pT7Asp.

Lane 1: DNA molecular weight marker (1 Kb step ladder).
Lane 2: PCR amplified complete asparaginase \{ansB (+) gene\} fragment (1047 bp).
Lane 3: Plasmid pRSET-A (un-digested).
Lane 4: Recombinant plasmid pT7Asp (un-digested).
Lane 5: Recombinant plasmid pT7Asp digested with \textit{NdeI} and \textit{BamHI}.
Fig 4.4A - Schematic representation showing the construction of the recombinant plasmid pR57Asp.
Fig 4.4B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pR57Asp.

Lane 1: DNA molecular weight marker (1 Kb step ladder).
Lane 2: PCR amplified mature asparaginase \( ansB \) (-) gene fragment (981 bp).
Lane 3: Plasmid pRSET-A (un-digested).
Lane 4: Recombinant plasmid pR57Asp (un-digested).
Lane 5: Recombinant plasmid pR57Asp digested with \( NdeI \) and \( BamHI \).
Fig 4.5A - Schematic representation showing the construction of the recombinant plasmid pTacAsp.
Fig 4.5B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pTacAsp.

Lane 1: DNA molecular weight marker (1 Kb step ladder).
Lane 2: Recombinant plasmid pTacAsp digested with NdeI and BamHI.
Lane 3: Plasmid pMAL-p2x (un-digested).
Lane 4: Recombinant plasmid pTacAsp (un-digested).
Lane 5: PCR amplified asparaginase \( ansB \) (+) gene fragment (1047 bp).
Fig 4.6A - Schematic representation showing the construction of the recombinant plasmid pRAspC.
Fig 4.6B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pRAspC.

Lane 1: PCR amplified asparaginase \{ansB (+) gene\} fragment with a C-terminal 6x histidine-tag (1100 bp).
Lane 2: Plasmid pRSET-A (un-digested).
Lane 3: Recombinant plasmid pRAspC (un-digested).
Lane 4: Recombinant plasmid pRAspC digested with \textit{NdeI} and \textit{HindIII}.
Lane 5: DNA molecular weight marker (1 Kb step ladder).
Fig 4.7A - Schematic representation showing the construction of the recombinant plasmid pNHAsp.
Fig 4.7B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pNHAsp.

Lane 1: DNA molecular weight marker (1 Kb step ladder).
Lane 2: PCR amplified asparaginase \( \{ \text{ansB} \} \) gene fragment (981 bp).
Lane 3: Plasmid pET-14b (un-digested).
Lane 4: Recombinant plasmid pNHAsp (un-digested).
Lane 5: Recombinant plasmid pNHAsp digested with \( NdeI \) and \( BamHI \).
Fig 4.8A - Schematic representation showing the construction of the recombinant plasmid pETxyl-22b.
**Fig 4.8B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pETxyl-22b.**

**Lane 1:** DNA molecular weight marker (100 bp step ladder).
**Lane 2:** PCR amplified *Bacillus* endoxylanase signal sequence fragment (~100 bp).
**Lane 3:** Plasmid pET-22b (un-digested).
**Lane 4:** Recombinant plasmid pETxyl-22b (un-digested).
**Lane 5:** Recombinant plasmid pETxyl-22b digested with *NdeI* and *NcoI*. 
Fig 4.9A - Schematic representation showing the construction of the recombinant plasmid pPNHAsp.
Fig 4.9B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid pPNHAsp.

**Lane 1:** PCR amplified asparaginase \( \{\text{ansB} (-) \text{ gene}\} \) fragment (981 bp).
**Lane 2:** Plasmid pET-22b (un-digested).
**Lane 3:** Recombinant plasmid pPNHAsp (un-digested).
**Lane 4:** Recombinant plasmid pPNHAsp digested with *NcoI* and *BamHI*.
**Lane 5:** DNA molecular weight marker (1 Kb step ladder).
PCR amplified \textit{ansB} (+) gene

**Fig 4.10A** - Schematic representation showing the construction of the recombinant plasmid p29Asp.
Fig 4.10B - 1% Agarose gel showing the construction and restriction digestion of recombinant plasmid p29Asp.

Lane 1: DNA molecular weight marker (1 Kb step ladder).
Lane 2: Plasmid pET-29a (un-digested).
Lane 3: Recombinant plasmid p29Asp (un-digested).
Lane 4: PCR amplified asparaginase \( \text{ansB (++) gene} \) fragment (1047 bp).
Lane 5: Recombinant plasmid p29Asp digested with \textit{NdeI} and \textit{BamHI}.
PCR amplified \textit{ans}B (-) gene

\textit{Nco I} – \textit{BamH I} digestion

Ligation

\textit{Nco I} – \textit{BamH I} digestion

\textsf{pETxyl-22b (5.5 kb)}

\textsf{pXylAsp (6.5 kb)}

\textbf{Fig 4.11A - Schematic representation showing the construction of the recombinant plasmid pXylAsp.}
Fig 4.11B - 1% Agarose gel showing the construction of recombinant plasmid pXylAsp.

**Lane 1:** DNA molecular weight marker (1 Kb step ladder).
**Lane 2:** PCR amplified asparaginase \(\text{ansB} (-)\) gene fragment (981 bp).
**Lane 3:** Plasmid pETxyl-22b (un-digested).
**Lane 4:** Recombinant plasmid pXylAsp (un-digested).
**Lane 5:** Recombinant plasmid pXylAsp digested with \(NcoI\) and \(BamHI\).
**PRELIMINARY EXPRESSION STUDIES**

*a) Shake-flask studies on intracellular (cytoplasmic) expression*

Expression vectors pR57Asp and pNHAsp were used to transform BL21 (DE3) cells. A single transformed colony from the petriplate was inoculated in 10 ml LB containing 1X (100 μg/ml) ampicillin and grown overnight at 37°C with continuous shaking at 220 rpm. 1 ml of this overnight grown primary culture was inoculated into 100 ml LB medium supplemented with ampicillin and incubated under similar conditions. The optical density of the cells at 600 nm (OD$_{600}$) was monitored throughout the cultivation period. At an OD$_{600}$ of 0.65 (mid log phase), the 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).

![Graph](Fig 4.12 - Growth profiles showing OD$_{600}$ and LnOD$_{600}$ (inset) over time of cultivation. a) pR57Asp vector and b) pNHAsp vector in BL21 (DE3) cells.)
The cell growth continued unabated even after IPTG induction in both the cases and a final cell density of 3.0 and 3.5 was achieved six hours post-induction in cases of pR57Asp and pNHAsp respectively (Fig 4.12 a & b).

High-level recombinant asparaginase expression was obtained in the cytoplasm, which remained high throughout the cultivation. Cells were lysed by sonication and almost all of the recombinant asparaginase expressed was found to be present in the form of insoluble aggregates (see Fig 4.17). The level of recombinant protein was measured by isolation of inclusion bodies (IB's) from 1 ml cell pellet and the protein aggregates were solubilized in 1% SDS and the protein was estimated by Bradford method (see material & methods). The protein yield was also crosschecked by gel densitometry using BSA as a quantitative marker for protein estimation (data not shown). The final expression yields of ~125 mg/L and ~105 mg/L of recombinant asparaginase were achieved in case of pR57Asp and pNHAsp respectively.

**Scale-up to fermentor level**

Fed-batch cultivation using pNHAsp/BL21 (DE3) cells was done in a 3-litre fermentor with a working volume of 1.2 litres. Ampicillin at 2X concentration was used both in batch as well as feed medium. The temperature, pH and DO were
set at 37°C, 7.2 and 40% respectively. The airflow rate was set at 1 vvm and was not changed during the run. For high cell density cultivation a two-stage cultivation strategy was adopted. In the first stage (batch phase) minimal media supplemented with Y.E (M9 + 0.5% glucose + 0.5% yeast extract) was used. At an OD₆₀₀ of 8.0, complex feed was attached to maintain a specific growth rate (μ) of 0.3 h⁻¹ by feeding concentrated media (15% yeast extract + 15% glucose). This value of μ was chosen from the previous optimization studies conducted in our laboratory and also from the literature reports of optimum μ values for recombinant protein expression (Yazdani and Mukherjee 1998; Sanden et al. 2003; Srivastava et al. 2005). Exponential feeding was used to allow the cells to grow at a constant specific growth rate. The culture was induced at an OD₆₀₀ of 22.5 with 1 mM IPTG.

A final OD₆₀₀ of 64.5 was achieved in 10 hours post-induction corresponding to a final biomass of ~ 24 g/L dry cell weight (DCW) was achieved. The recombinant product profile was monitored on a SDS-PAGE gel by taking hourly samples (Fig 4.15). A final product yield of ~
4.0 g/L of recombinant asparaginase was obtained 10 hours post induction which corresponds to ~ 35% of the total cell protein. This level of expression compares favorably with the cytoplasmic expression levels of the other recombinant proteins of therapeutic interest (Srivastava and Mukherjee 2005; Yamasaki et al. 1998; Shibui et al. 1993).

Purification studies (shake-flask level)

Purification and column refolding by Ni-NTA affinity chromatography: The recombinant protein expressed as inclusion bodies was purified and refolded into active form using a solid phase refolding strategy. Refolding using such packed columns is attractive because it is easily automated using commercially available preparative chromatography systems i.e. scale-up is easier. The basic approach followed here was, immobilization of the denatured protein onto the matrix and subsequent denaturant dilution to promote folding. The aim here was to separate individual protein molecules spatially, thus inhibiting aggregation (Rogl et al. 1998; Zahn et al. 1997) and since the protein molecules were bound to the resin, this procedure minimized the aggregation of the unfolded protein or the folding intermediates (Tsumoto et al. 2003). Affinity interactions are also preferred over other immobilization through non-specific interactions (like size exclusion and ion exchange columns) because these allow binding through specific domains, with the bulk of the protein free from the surface and hence able to refold (Middelberg APJ 2002). Another considerable advantage is that folding could be conducted at significantly higher protein concentrations than those achieved in dilution refolding.

For this pNHAasp / BL21 (DE3) cells were harvested after six hours post-induction where the recombinant asparaginase was expressed as a fusion protein with a cleavable N-terminal 6 x histidine tag. The cell pellet from 200 ml of the induced culture (containing ~ 10.5 mg of recombinant protein) was used for column refolding and purification. The cell pellet was lysed in 10 ml of solubilization buffer (50 mM Sodium Phosphate buffer pH 7.4 containing 300 mM NaCl, 20 mM
imidazole and 8M urea) and incubated for 1 hour on a rotary shaker at room temperature to solubilize the total cellular proteins. The suspension was centrifuged at 14,000 x 'g' for 1 hour at room temperature and the supernatant was collected and passed through a 22 G (gauge) needle in order to shear the genomic DNA, which also reduces the viscosity of the buffer-protein suspension. The supernatant was then filtered through a 0.22-micron disposable filter and then loaded on to a Ni-NTA column pre equilibrated with solubilization buffer. The loaded column was then washed with 4 column volumes of solubilization buffer. This was followed by a gradient refolding procedure (Schauer et al. 2003; Gupta P et al. 1999), where the urea concentration was reduced in a stepwise fashion with reduction of 1M-urea concentration in each step (from 8 M to 0 M urea). The column was then washed again with 2 volumes of washing buffer (solubilization buffer without urea and NaCl). Finally bound asparaginase was eluted from the column with elution buffer (50 mM Sodium Phosphate buffer pH 8.0 containing 250 mM imidazole).

The eluted fractions were analysed on a SDS-PAGE and also estimated for presence of protein by Bradford dye method. The desired fractions were pooled and dialysed against sodium phosphate buffer pH 8.0 in order to remove imidazole. The protein sample after dialysis was collected and centrifuged at

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**Fig 4.16** - Purification and refolding of recombinant asparaginase by Ni-NTA purification from pNHAsp/BL21 (DE3) cells. 250mM imidazole concentration was used for elution of bound protein.
12,000 x ‘g’ for 10 minutes for the misfolded and thus aggregated protein molecules to settle down and the supernatant was analysed for asparaginase activity.

Fairly pure protein was obtained by column refolding (Fig 4.16), with a reasonably high recovery of ~ 70% of the recombinant protein after the imidazole elution step (7.5 mg of asparaginase recovered out of 10.5 mg present in the cell extract). However, poor final recombinant asparaginase yield was obtained in this case, with the major problem being aggregation of the refolded protein during the dialysis step. More than 65% of the total purified protein aggregated (giving appearance as clumps of cotton) and a final protein yield of 1.05 mg was recovered in the active form, which corresponds to a final recovery of ~ 10%.

It was felt that loading the total cell lysates onto the column could be a problem. The presence and interference of the other cellular proteins during the solubilization and refolding could be a reason for the poor refolding yield. It was therefore decided to first isolate inclusion bodies and then solubilize and refold it on a Ni-NTA column.

**Inclusion body isolation**: The inclusion bodies obtained by overexpression of recombinant proteins in the cytoplasm are large amorphous protein aggregates that because of their refractile character, can be observed directly in the living host by phase contrast microscopy. Inclusion bodies are usually released mechanically or chemically from the cell (Middelberg 1995) followed by centrifugation or filtration. Generally isolated inclusion bodies have been shown to contain contaminating polypeptides, phospholipids and traces of nucleic acids (Valax and Georgiou 1993). There is increasing evidence that these contaminants can significantly reduce the refolding yield (Maachupalli-Reddy et al. 1997). Hence inclusion body isolation and purification was done prior to the column-refolding step, to check whether the interference of the cellular proteins from *E. coli* could be the cause for poor refolding yields.
For isolation and purification of inclusion bodies, the pNHAsp/BL21 (DE3) cells were harvested after six hours post-induction. The cell pellet from 200 ml of induced culture (containing ~ 10.5 mg of recombinant protein) was used for isolation and purification to generate a reasonably pure inclusion body preparation. The cell pellet was resuspended in 10 ml of 50 mM sodium phosphate buffer, pH 7.5 and sonicated to ensure complete lysis. The lysate was centrifuged at 14,000 x 'g' for 20 minutes. The pellet consisting of the insoluble IB's was resuspended in 15 ml of 50 mM phosphate buffer pH 8.0, containing 1M urea and 300 mM NaCl. This was incubated for 15 minutes on a rotary shaker. This step was done to selectively solubilize the cell debris and other associated proteins without affecting the IB's (urea > 2 M tends to solubilize the inclusion bodies and thus would result in major recombinant protein losses at this step; data not shown). The suspension was centrifuged as earlier and the supernatant was discarded. This step was repeated. The pellet was then washed twice with ddH2O and resuspended in 20 ml of 50 mM phosphate buffer pH 8.0 and deoxycholic acid (DOC) was added to a final concentration of 2 mg/ml. The suspension was incubated on a rotary shaker for 1 hour and centrifuged at 14,000 x g for 10 minutes. This step was also repeated and the final pellet was repeatedly washed with ddH2O to remove deoxycholic acid. All these steps essentially constituted the washing steps designed to get rid of impurities that get associated with the IB's (Fischer et al. 1993). Thus fractions collected at various steps were analysed on a 12% SDS-PAGE gel for protein impurities (Fig 4.17). A single protein band was observed on the gel after the final wash suggesting that
a fairly pure asparaginase preparation with a recovery of 70% had been obtained. The inclusion bodies were then solubilized in 10 ml of 25 mM Tris HCl buffer, pH 8.0 containing 8M urea (with and without 2 mM β-ME) by incubating it for 40 minutes. The suspension was centrifuged at 14,000 x 'g' for 30 minutes to get rid of cell debris. The supernatant was carefully decanted and stored in a falcon tube. The supernatant was passed through a sterile disposable 0.22-micron filter (low protein binding) to remove any particulate matter that may have come from cell debris. The concentration of recombinant asparaginase present in the protein filtrate was determined spectrophotometrically to be 7.2 mg using the following extinction coefficients: at 276 nm for protein in 8M urea, \( E_{276}^{1\%} = 6.5 \); and at 278 nm in buffer solution, \( E_{278}^{1\%} = 7.1 \) (Frank et al. 1970). The filtrate was diluted appropriately to reach a final asparaginase concentration of 0.25 mg per ml (maintaining the urea concentration at 8 M). This was loaded onto the Ni-NTA column and washing and refolding was followed (as described in the previous experiment).

In this case, the final yield of the active recombinant asparaginase achieved was 0.8 mg which meant that ~ 12% recovery after the IB purification step. In short there was almost negligible improvement in the refolding efficiency by removal of the contaminating cellular proteins prior to refolding rather there was small decrease in the final product yield by addition of the inclusion body purification step. This clearly demonstrated that the aggregation of recombinant asparaginase was not due to the presence of contaminating proteins rather it was an intrinsic property of the protein itself.

Attempts at reducing this aggregation problem at the dialysis step were followed, like reducing the concentration of eluted protein by 10 folds (0.1 mg/ml) prior to dialysis to remove imidazole (in order to minimize the formation of wrong aggregates, which can show concentration dependence; Jaenicke and Rudolph 1986) and also by dialyzing the protein in presence of glycerol (up to 10 % v/v) (glycerol tend to stabilize the native structure of globular proteins by preferential hydration; Gekko and Timasheff 1981; Mishra et al. 2005) but using these
methods did not increase the final yield of active protein beyond ~ 15%. Presence or absence of β-ME during solubilization of inclusion bodies did not result in improving the final product yield.

Additionally, a few dilution-refolding studies were also done involving the non-tagged asparaginase. The purified inclusion bodies were solubilized as described in earlier experiments and the protein was refolded by diluting it into 25 mM Tris HCl buffer pH 8.0 at 25°C. From the stock various amounts of denatured protein were diluted into refolding buffer such that the final concentration of recombinant asparaginase was in the range of 25 μg/ml to 500 μg/ml. Denatured protein was added drop wise into the buffer which was kept at constant stirring at 25°C. Disulphide bond formation was facilitated by air oxidation (Ahmed et al. 1975; Fischer et al. 1993) since the asparaginase monomer in the native form has both the cysteine residues coupled in the single intrachain disulphide bond form. The reaction was catalyzed by the presence of 10 μM CuSO₄ (final concentration) (Menzella et al. 2002). The refolding mixture was incubated for an optimized time of 24 hours and then assayed for asparaginase activity. It was observed that at lower concentrations (25 - 50 μg/ml) a modest refolding efficiency of 15% was achieved, which fell further with the increase in protein concentration (data not shown).

Pulse addition of denatured protein (50 μg/ml per addition) was also done, pulse addition was done every 2 hours, and the refolding mixture was incubated for 24 hours prior to assay. It was observed that pulse addition of lower denatured protein concentration didn’t result in achieving a high refolding yield at higher protein concentrations (data not shown).

Although these refolding studies were not extensive enough to make the most comprehensive set, there are sufficient indicators to point towards need for more complex refolding strategies probably involving the oxido-shuffling system for the formation of disulphide bond (Rudolph and Lilie 1996) and/or addition of chemical chaperones to assist refolding by resisting the aggregation of unfolded and the intermediate forms (Rozema and Gellman 1996; Nath and Rao 2001; Machida et al. 2000). Such systems though useful at the lab-scale, are not commercially
viable given the high cost of some of these reagents, such as oxidized glutathione (Clark 1998).

In order to understand the difficulties associated with getting high refolding yields it is important to look at the structure of native asparaginase protein. Asparaginase in its native form is an oligomeric protein (homotetramer) and additionally the monomer itself is a two-domain protein (see fig 2.4; literature review). Each subunit consists of two $\alpha/\beta$ domains connected by a linking sequence (residues 191 - 212). The N-terminal domain is the larger one (residues 23 - 190), which contains an eight-stranded mixed $\beta$ sheet. The smaller C-terminal domain (residues 213 - 326) consists of a four-stranded parallel $\beta$ sheets and four $\alpha$ helices. Location of the four active sites is between the subunits in the intimate dimers. Each dimer has two active sites, although only the tetramer has been reported to show activity. Each active site is made up primarily of residues from the N-terminal domain, which contacts from the C-terminal domain of the intimately bound subunit (Swain et al. 1993).

For oligomeric proteins, the yield and rate of reconstitution of native form depends on the mode of denaturation, as well as the solvent conditions and protein concentration during renaturation. The most prominent side reaction competing with reconstitution is the formation of “wrong aggregates” generated by weak molecular interactions of groups which in the native molecule tend to be involved with intramolecular or interdomain contacts. The effect becomes increasingly important with increasing size of protein subunits, because folding by parts then dominates, giving rise to wrong interactions (Jaenicke and Rudolph 1986).

For larger multi-domain or multi-subunit proteins the rate of refolding can be much slower and the efficiency of the process relatively low, with the formation of aggregates a common competing process (Kelly and Price 1991).

Thus the tendency to aggregate on renaturation, which we observed with asparaginase, was primarily to do with its complex structure. Though no report on refolding of asparaginase from inclusion bodies exists, a report on refolding of a single-chain Fv-Asparaginase fusion protein (where the chimeric enzyme
formed inclusion bodies when expressed in *E. coli* BL21 (DE3) cells and did not exhibit catalytic activity). Inclusion bodies could not be refolded to active form this case. Moreover, the active asparaginase fusion protein that was purified from the small soluble fraction (which was 2.3% of the total soluble protein fraction) was present as a dimer and not in the native form (Guo et al. 2000). This study in some ways supports our view, as attempts to refold the recombinant fusion protein failed miserably in this case as well. Thus though we observed an extremely high product yield with cytoplasmic expression, it was felt that *in vitro* refolding would become a major bottleneck in the design of a comprehensive bioprocess strategy for asparaginase production. It was therefore decided to work for expression of correctly folded asparaginase which was possible only with secretory expression.

**b) Preliminary expression studies for extracellular asparaginase expression**

In order to check the efficacy of plasmid based recombinant asparaginase expression the background asparaginase expression levels in the various *E. coli* host strains to be involved in the study need to be checked and measured.

![Fig 4.18](image-url) - Analysis of background asparaginase expression from the various host cells used in expression study. T - TB1; BL - BL21 (DE3); BR - BLR (DE3); ER - ER2508; +ve - induced PNHAsp cells. a) 12% SDS-PAGE analysis, b) Western blot analysis.
For this, the expression strains {TB1, BL21 (DE3), BLR (DE3) and ER2508} were grown in TB media in shake-flasks for 24 hours. The pT7Asp/BL21 (DE3) cells were also cultured and induced, and were considered as positive control for asparaginase expression. Culture samples were collected and centrifuged to obtain cell pellets and culture supernatants. All the samples were run on a SDS-PAGE gel and subjected to western blot analysis to detect asparaginase levels in the media as well as inside the cell. As is evident from the gel picture and western blot analysis (Fig 4.18), all the *E. coli* expression strains showed no detectable asparaginase expression levels in the extracellular space as well as inside the cells. Only the positive controls (extracellular and intracellular) lighted up in western blot.

**Localization of recombinant asparaginase:** The pT7Asp/BL21 (DE3) cells were grown in TB medium and induced with 0.5 mM IPTG at an OD$_{600}$ of 2.0. Cell pellets and culture supernatants were collected at defined time intervals after induction. Recombinant asparaginase present in the culture media comprised the extracellular fraction and the cell pellets were processed to obtain periplasmic fraction and cytoplasmic fraction (see materials & methods).

![Fig 4.19 - SDS-PAGE analysis of localization of recombinant asparaginase in induced T7Asp cells at various hours post-induction. a) Extracellular b) Periplasmic and c) Cytoplasmic fraction. Arrows indicate position of recombinant asparaginase in gel picture.](image)
All the fractions were run on a SDS-PAGE gel to assess the relative fractions of recombinant asparaginase present in each compartment. As is evident from the gel analysis, there was a constant increase in the level of asparaginase in the extracellular fraction, while the periplasmic expression levels were more or less constant. The cytoplasmic expression levels were even lower. The largest fraction of recombinant asparaginase was obtained in the extracellular space. The time profiles also indicated towards efficient transport of the recombinant protein from the cytoplasm to the periplasmic space and then from there to the extracellular space.
EXPRESSION STUDIES (SHAKE-FLASK LEVEL)

Effect of IPTG concentration on extracellular asparaginase expression

The effect of varying the concentration of the inducer IPTG on growth and product formation in the extracellular culture media was studied. For this E. coli BL21 (DE3) cells transformed with recombinant plasmid pRT7Asp were grown overnight in a 100 ml flask containing 10 ml LB. This culture was used to inoculate three separate 500 ml flasks containing 50 ml TB. Growth was monitored by measuring optical density at 600 nm (OD$_{600}$), and the cells were induced at an OD$_{600}$ of 2.0-2.5 (mid-log phase of growth) with different IPTG concentrations of 0.1 mM, 0.5 mM and 1.0 mM. Samples were collected at various time points post-induction, cells were pelleted and the supernatant was assayed for asparaginase activity.

As is evident from the growth profile (Fig 4.19a), increasing concentrations of IPTG result in suppression of cell growth thus adversely affecting the final biomass yield.

The maximum inhibition of cell growth occurred within 2 hours post-induction in a concentration dependent manner demonstrating the stress associated with recombinant protein expression. A decrease in the rate of product secretion and

Fig 4.19 a) Growth profile (OD$_{600}$) and b) Activity profile (Units/ml) of recombinant pT7Asp vector/BL21 (DE3) cells for extracellular asparaginase expression over time.
therefore a decline in the final amount of product secreted to the extracellular media was also observed with increasing concentrations of IPTG (Fig 4.19b). Clearly the adverse effect of excess IPTG on growth also impacted on product formation kinetics, suggesting that induction with low concentration of 0.1 mM of IPTG was optimal for attaining a higher growth and asparaginase yields in the culture media. By running a SDS-PAGE, it was observed that very few contaminating proteins were present along with asparaginase in the extracellular medium (Fig 4.20).

Optimization of the best host-vector combination for asparaginase expression

Vector optimization: In order to check the relative efficacy of signal sequences, promoter and the effect of different fusions, expression plasmids constructed earlier (Table 3.2) were transformed individually into BL21 (DE3) cells and checked for cell growth and protein expression. For this cells were grown overnight in 100 ml flasks containing 10 ml LB and these primary cultures were used to inoculate 500 ml flasks containing 50 ml TB. Growth was monitored by measuring $OD_{600}$, and the cells were induced at an $OD_{600}$ of 2.0-2.5 (mid-log phase of growth) with 0.1 mM IPTG. Samples were collected at various time points post-induction as described previously.

All the recombinant cultures had different growth profiles resulting in the attainment of different biomass concentrations. In the case of TacAsp cells, T7Asp cells and RaspC cells, growth was hardly affected upon induction and a final biomass corresponding to $OD_{600}$ of 13-14.5 was obtained. Lowest cell
densities were obtained in the case of PNHAsp and 29Asp cells (OD<sub>600</sub> of 8.5 – 9.0) where suppression in cell growth was observed a few hours after IPTG induction. XylAsp cells gave an intermediate value of the final biomass concentration (Fig 4.21a).

An inverse correlation was observed between the growth and activity profiles. Asparaginase secretion to the culture media increased continuously over time. Maximum volumetric activity of 17.6 Units/ml was obtained with PNHAsp cells which was more than 3 fold higher to that of XylAsp cells and RA.SpC cells. The remaining clones gave intermediate values of extracellular expression. Thus PNHAsp cells showed the maximum activity while the RA.SpC cells had the poorest (Fig 4.21b). This was an expected result since it is well known that there is a metabolic burden associated with the recombinant protein expression that would tend to increase with higher expression levels.

Since the level of expression increased continuously to reach a maximum at 24 hours, the 24<sup>th</sup> hour post induction samples were used to compare the level of secretory expression as well as the efficiency of export of recombinant asparaginase to the extracellular space. For this samples were analysed for extracellular and periplasmic levels of recombinant asparaginase expression (Fig 4.22).
The comparative levels of expression obtained in case I and case II demonstrates that cloning the asparaginase gene under the T7 promoter results in ~ 22% higher levels of total secretory expression (i.e. periplasmic and extracellular fractions taken together) in comparison to the tac promoter. Since the export efficiency (from the periplasmic space to the extracellular compartment) for both the promoters was found to be similar, higher levels of recombinant asparaginase were obtained in the culture media in case II. The effect of the antibiotic selection marker on secretory expression was examined by comparing case II and case IV. The total secretory expression was higher by ~ 32% in case of ampicillin selection in comparison to kanamycin, though the efficiency of export was slightly better with the kanamycin marker. The recombinant protein was histidine tagged for ease of purification, at both ends separately. Histidine tag at the C-terminus (case III) had no effect on total secretory expression, but it did interfere with the export efficiency to the culture media which declined to 50% in comparison to non-tagged protein as seen in case II. N-terminal histidine tag on the other hand (case V) did not have any inhibitory effect on protein export to the culture media.

The effect of signal sequences on the total secretory expression and export efficiency to the media was also checked. Comparative analysis of the asparaginase native signal sequence (case II), pelB signal sequence (case V) and Bacillus endoxylanase signal sequence (case VII) showed that total secretory expression was highest when the pelB signal sequence was used. This value was 50% higher than that of Bacillus endoxylanase signal sequence and 17% more than
the native signal sequence. Extracellular export efficiency was also higher when pelB was used with ~ 63% of recombinant protein being exported to culture media. In comparison the *Bacillus* endoxylanase signal sequence gave a much lower secretory expression that was only ~ 53% of the values obtained with the pelB signal sequence. The exported fraction was also lower at 40%. Case VI was used as a non-secretory control where the BL21 (DE3) cells were transformed with the expression vector containing only the mature asparaginase gene without any signal peptide. The asparaginase protein in this case accumulated almost exclusively in the form of inclusion bodies in the cytoplasm (as reported earlier). Very low activity was observed in the periplasmic fraction and the culture media during the final stages of cultivation. We thus observed that BL21 (DE3) cells transformed with the recombinant plasmid pNHAsp containing the asparaginase gene with a pelB leader sequence and a N-terminal histidine tag under the T7 *lac* promoter gave the best result in terms of total secretory production and extracellular secretion efficiency. This vector was therefore selected for further studies involving host selection.

**Host-optimization:** The recombinant pPNHAsp expression vector was transformed into a series of *E. coli* expression hosts to obtain the best host-vector combination for expression studies. Five *E. coli* expression hosts were considered viz - TB1 strain (for basal level expression), ER2508 (a lon protease deficient expression strain) and three T7 based expression strains viz - BL21 (DE3), BL21star (DE3) and BLR (DE3) (Table 3.2). Cells were grown overnight in 100 ml flasks containing 10 ml LB medium. These primary cultures were used to inoculate 500 ml flasks carrying 50 ml TB medium each carrying 100 μg/ml of ampicillin. Growth was monitored by measuring OD₆₀₀, and the cells were induced at OD₆₀₀ of 2.0-2.5 (mid-log phase of growth) with 0.1 mM IPTG. The culture media samples were collected at various hours in the post-induction period, cells were pelleted and supernatant was collected as extracellular fraction and assayed for asparaginase activity.
Here again we observed an inverse correlation between growth and expression level. Cells that gave high levels of asparaginase expression grew poorly in the post induction period.

Thus recombinant TB1 cells which continued to grow unabated throughout the cultivation period with no repressive effect of IPTG induction being observed, had the poorest expression levels even though highest final biomass level corresponding to an OD$_{600}$ of 24 was achieved 24 hours post induction (Fig 4.23a). In all other cases repression of growth was observed and intermediate levels of final biomass levels in the range of 11 – 15 were obtained. In the case of BLR (DE3) cells, where the lowest cell biomass levels were obtained corresponding to an OD$_{600}$ of 9.0 the expression levels were the highest.
The activity profiles (Fig 4.23b) showed a continuous increase in extracellular asparaginase secretion over time in all the three T7 based expression systems. In recombinant TB1 and ER2556 cells a plateau in asparaginase activity was reached within 16 hours post-induction. Very low expression levels corresponding to ~ 2 Units/ml and ~ 7 Units/ml were thus reached in cases of recombinant TB1 and ER2556 cells respectively. Recombinant BLR (DE3) cells gave the highest extracellular levels of 22.5 Units/ml, whereas recombinant BL21 (DE3) and BL21star (DE3) cells resulted in intermediate extracellular asparaginase expression levels. Since the level of expression increased continuously to reach a maximum at 24 hours, the 24th hour post induction extracellular and periplasmic samples were analyzed for recombinant asparaginase production (Fig 4.24).

The total secretory expression level was minimum in the case of TB1 cells (~ 6 Units/ml) and the export efficiency was 50%. The total secretory expression level and efficiency of secretion increased by 44% and 69% respectively in the case of ER2508 cells. The levels of protein expression increased significantly in the three T7 based expression hosts. In case of BL21 (DE3), which is, the standard host used in expression studies, 25.3 Units/ml of total secretory expression was achieved out of which ~ 62% was exported to the media, while with BL21star (DE3) cells the total secretory expression as well as the export efficiency was slightly higher. The maximum total secretory expression was achieved in BLR (DE3) host with a production of 30.67 Units/ml of which ~ 75% of the recombinant protein was exported into the media. Therefore the host-vector combination of pNHAsp vector and BLR (DE3) strain was used in subsequent studies.

Effect of media composition on extracellular asparaginase expression

The PNHAsp cells {i.e. the recombinant BLR (DE3) cells transformed by the pPNHAsp expression vector} were grown to mid log phase in different media i.e. LB, 2x YT and TB supplemented with ampicillin and then induced at mid-log
phase of growth with 0.1 mM IPTG. The cultures were grown for 24 hours post induction, samples were collected at different time points and the supernatants analyzed for asparaginase activity and total protein content. In 2x YT medium the final biomass achieved (OD$_{600}$ of 4.5) was 1.5 fold higher than that achieved in LB (OD$_{600}$ of 3.0), while the highest biomass was achieved in TB (OD$_{600}$ of 8.0). It was clearly evident from the growth profile that use of rich media resisted the decline in growth in the post induction period better than in LB. In TB the specific growth rate remained constant at its pre-induction value for 2 hours post induction after which it showed a steady decline over the next few hours. In 2x YT and LB medium there was a sharper decline in the specific growth rate ($\mu$) within one-hour post induction, indicating that the availability of complex nitrogen sources in excess help in reducing the stress associated with IPTG induction (Fig 4.25a).

The activity profile showed a steady increase indicating the buildup of recombinant asparaginase in the media, with TB giving the maximum extracellular secretory expression (Fig 4.25b).

Since the level of expression increased continuously to reach a maximum at 24 hours, the 24th hour post induction extracellular samples were analyzed for comparing the recombinant asparaginase expression. The maximum volumetric
activity was observed in TB, which was 4 fold higher to LB and 2.3 fold higher than that of 2x YT media. However when the specific productivity (activity/OD$_{600}$) was compared no significant differences were observed demonstrating that the productive ability of the individual cell was not affected by the media constituents. Thus higher expression level in TB was primarily obtained by increasing the biomass concentration in the culture. The specific activity (activity/total protein concentration in the media) which is a measure of specificity of recombinant protein secretion and indicates the presence or absence of other contaminating proteins, was highest in LB showing that rich media also promotes non-specific secretion. However given its ability to give high biomass concentration and hence higher volumetric activity (Fig 4.26), TB was chosen for further studies in order to improve secretion of recombinant asparaginase.

**Effect of OD$_{600}$ of induction on extracellular asparaginase expression**

The PNHAsp cells were induced at different stages of growth in order to determine the optimum time point of induction. For this PNHAsp cells were grown in TB and induced with 0.1 mM IPTG at different cell densities of OD$_{600}$ = 0.6 (early log), OD$_{600}$ = 2.0 (mid log) and OD$_{600}$ = 4.5 (late log) stage of growth. The cultures were grown for 24 hours post induction, samples were collected at different time points and the supernatants analyzed for asparaginase activity and total protein content (Fig 4.27 a & b).
Induction at early log stage resulted in a high final cell density $OD_{600}$ of 10.0, which was comparable to the un-induced control culture ($OD_{600}$ of 10.8). However IPTG induction did result in the characteristic suppression of growth which was lost ~ 10 hours post-induction after which the cells grew fairly fast to reach cell densities compared to the un-induced control culture. Induction at the late log phase resulted in the maximum and permanent suppression of growth and there was negligible increase in cell density post-induction, which is indicated by the flattening out of the growth curve. The final cell $OD_{600}$ was 5.5, which was nearly half to that of early induction. Induction at a mid log phase yielded intermediate values of cell densities, a partial growth suppression and a final cell $OD_{600}$ of 8.0. The un-induced culture showed an uninhibited growth pattern characteristic of $E. coli$ cultivation in shake-flasks, with the specific growth rate falling towards the later stage of cultivation probably due to nutrient limitation (Fig 4.27a).

A steady build up in the recombinant asparaginase levels in the media was observed in all three cases. The highest volumetric activity of 20.2 Units/ml was observed in case of delayed induction (late log phase), which was ~ 1.6 fold higher to that achieved in case of an early log phase induction. Induction at the
mid log phase resulted in the achievement of an intermediate volumetric activity of 16 Units/ml (Fig 4.27b).

The recovery of growth when the cells were induced early coupled with the lower levels of expression obtained clearly indicates loss of expression ability of these cells in the later part of cultivation period. This could either be due to the emergence of non-recombinant cells in the culture (plasmid instability) or loss of induction pressure (degradation of IPTG). Given the small number of generations for which cells grow in a shake-flask the likelihood of plasmid instability seems remote.

The most interesting observation was that the rate of recombinant protein expression continued unhindered even when the cell growth was stopped. The increase in product concentration in the extracellular broth slowed down only after 15 hours of induction possibly due to nutrient starvation. This observation provided us with the key to design fed-batch strategies for high-level expression (described later in chapter 5).

Since the level of expression increased continuously to reach a maximum at 24 hours, the 24th hour post induction extracellular and periplasmic samples were analyzed for recombinant asparaginase expression. We observed that not only were the volumetric activities high also the specific productivity (total activity/OD_{600}) was almost 3 folds with late log phase induction (3.80 Units/OD_{600}) in comparison to early log phase induction (1.31 Units/OD_{600}). This clearly shows the loss of expression in the later part of the cultivation for the cells which were induced at early log phase. The specific activity was also slightly higher showing
that the total amount of the protein in the culture media did not increase proportionally to the increase in activity. These results suggest that specific secretion of the recombinant asparaginase was slightly better when induction at late log phase was done (Fig 4.28).

Western blot analysis of the recombinant asparaginase fractions present in the three E. coli compartments viz - extracellular, periplasm and the cytosol (during various hours of post-induction period) presented some interesting observations. There was a steady accumulation of recombinant asparaginase in the media over time in all three cases (early, mid and late OD of induction), which tended to stabilize towards the 20 - 24 hour post induction period (Fig 4.29).

However from the time profile of the periplasmic fraction we observe a steady decline in asparaginase levels with time in the late log phase induction demonstrating efficient transport across the outer cell membrane unlike the other two cases.

Also the periplasmic asparaginase build up in the fourth hour is better demonstrating efficient secretion from cytosol to periplasm. Efficient secretion is confirmed by looking at the cytosolic fraction where the fraction of processed
protein (lower band) is thicker in late log phase induction unlike the other two cases. The biggest problem with early and mid log phase induction is possibly the fast protein production kinetics leading to protein build up in the cytoplasm given that the processing and secretion of asparaginase is at a slower rate. This would adversely affect cellular health and increase the stress associated with the build up of recombinant protein in the cytoplasm. Under ideal circumstances all rates; that of expression and secretion (to the periplasm) and export should match each other. However in the later hours due to decline in expression (E & M cases) the cytoplasmic expression levels also fall.

**Plasmid stability analysis:** Plasmid instability was investigated primarily to see whether it was a cause for a) recovery in growth a few hours after induction, b) the lowering of specific productivity in case of early induction. The time point samples especially 16 hours post-induction onwards was analysed. For this PNHAsp cells were induced at different stages of growth in TB and induced with 0.1 mM IPTG at early (OD\textsubscript{600} = 0.6), mid (OD\textsubscript{600} = 2.0) and late log phase (OD\textsubscript{600} = 4.5) of growth.

The cultures were grown for 24 hours post induction and samples were collected at various time points, appropriately diluted and aliquots plated on LB agar plates without antibiotics. Normally a dilution of 10\textsuperscript{4} in saline was done and 100 μl of this was plated. On an average 100 isolated colonies were picked up from the LB agar plate by toothpicks and transferred on to LB-ampicillin plates. The fraction of the colonies that did not grow on the antibiotic plate gave the fraction of non-recombinants in the cell population.
It was observed that the plasmid remained reasonably stable up to 24 hours post-induction in all the three cases (Fig 4.30). The control culture (i.e. which was not induced throughout the cultivation) showed a plasmid stability of 94%. The stability declined in the induced cultures where predictably the highest stability was observed with late log phase induction (80%) to mid log phase induction (75%) and finally to 72% stability in early log phase induction. However these values of plasmid stability are not significantly different to explain the lower values of asparaginase expression or growth recovery and higher final biomass achieved in cases of early and mid log phase induction where the final biomass achieved was almost twice to that of late log phase induction. It should however be noted that we were measuring only the segregational instability of the plasmid.

Gel densitometry analysis: To check the efficiency of export 24-hour post-induction samples of the supernatant and total cell protein from late log phase induced culture was run on a SDS-PAGE. This confirmed the earlier result that 24 hours post induction ~ 75% of the total recombinant asparaginase had been secreted into the media in the case of late OD induction (Fig 4.31).

Effect of asparaginase expression on the morphology of E. coli: To directly visualize the health of cells by looking at the E coli cell morphology and to check whether cell lysis had contributed for increased secretion, the recombinant cells were stained and visualized by electron microscopy. For this, the PNHAsp cells were grown in TB and induced with 0.1 mM IPTG at mid (OD_{600} = 2.0) and late
(OD$_{600}$ = 4.5) log phase of growth. Cells were grown for 24 hours post-induction. Pre-induction and post-induction samples were collected. Cells were appropriately diluted and negatively stained with uranyl acetate solution for electron micrography (see material & methods). There was no apparent alteration in the appearance of the un-induced and the induced cells, and the smooth outer surface of cells indicate the presence of an intact outer cell membrane (Fig 4.32). Thus the possibility of non-specific leakage due to damage in the outer cell membrane was ruled out.

![Fig 4.32](image)

**Fig 4.32** - Electron micrographs of negatively stained PNHAsp cells collected after 24 hours of induction. a) Un-induced cells, b) cells induced at mid log phase and c) cells induced at late log phase.

Induction at the late log phase resulted not only in higher volumetric activity but more importantly promoted secretion specificity, where the recombinant protein constituted a major fraction of the total protein present in the extracellular medium. Typically high expression levels are correlated with high specific growth rates (Sanden et al. 2003; Shokri et al. 2002). However there are a few reports published recently that supports our findings. Ou et al. 2004 showed that stationary phase protein over-expression is a fundamental characteristic of *E. coli*, and fairly high-level expression of the target protein was achieved with induction at the stationary phase. Similarly higher solubility of the recombinant protein and lower proteolytic rates were reported with late log phase and stationary phase induction which resulted in enhanced recombinant protein yields (Galloway et al. 2003; Chae et al. 2003). In the present study, we also observed that induction in the late log phase led to protein over expression and facilitated
secretion, where the metabolic fluxes were directed primarily towards product formation. Thus growth arrest was observed one-hour post induction and the final OD$_{600}$ obtained was lower with late log phase induction than with early log phase induction.

**Effect of ampicillin variation on extracellular asparaginase expression**

Varying ampicillin concentrations were used for culture cultivation in order to see the effect of increased selection pressure on the recombinant both in terms of growth and extracellular asparaginase production. A higher concentration of ampicillin should enhance the copy number of plasmid (since it is under relaxed copy number control). Moreover β-lactamase is secreted into the culture space by the recombinant cells in substantial amounts, where it degrades ampicillin and thus allows the plasmid-free cells to grow. Thus in cases where cultivation periods are longer than the normal 4-6 hours post-induction, ampicillin degradation either due to β-lactamase secretion or hydrolysis under the acidic media conditions can be more prevalent and could easily lead to overtaking of the cell population by plasmid free cells. This in turn would affect the recombinant protein yield.

For this PNHAsp cells were grown overnight in a 100 ml flask containing 10 ml LB, and was used to inoculate four separate 500 ml flasks carrying 50 ml TB medium each carrying increasing concentrations of ampicillin viz - 100 μg/ml (1X), 200μg/ml (2X), 500 μg/ml (5X) and 1000 μg/ml (10X). Growth was monitored by measuring OD$_{600}$, and the cells were induced at OD$_{600}$ of 2.0-2.5 with 0.1 mM IPTG. Samples were collected at various time points post-induction, cells were pelleted and supernatant was collected as extracellular fraction and assayed for asparaginase activity.

Doubling of ampicillin concentration (2X) in the culture media did not have any affect on the growth while there was a marginal increase in the extracellular asparaginase expression. Increasing the ampicillin concentration five-fold (5X) did produce a decline in growth as well as product formation.
However the use of ten-fold (10X) ampicillin inhibited cell growth and this in turn resulted in poor expression levels and lower biomass yields (Fig 4.33 a & b). Thus while ampicillin is required for better expression either by increasing copy number or plasmid stability it needs to be present in reasonable amounts only so as not to affect growth and cellular health. The results also confirm indirectly that the plasmid is segregationally relatively stable.

**Effect of carbon source (glycerol / dextrose) on extracellular asparaginase expression**

Another possible reason for increased extracellular expression and secretion of recombinant asparaginase protein to the media in TB medium over the other two media could be because of the presence of glycerol, which is a relatively slowly utilizable carbon source by *E. coli*. On the other hand dextrose is a more readily metabolizable carbon source generally helping in the achievement of higher growth rates.

The effectiveness of the two carbon sources in terms of achieving higher biomass and recombinant asparaginase productivity in the media was compared. For this PNHAsp cells were grown overnight in a 100 ml flask containing 10 ml
LB medium. This culture was used to inoculate two 500 ml flasks, one carrying 50 ml TB with 0.4% glycerol and the other having TB with 0.4% dextrose. Both the cultures were grown in 2X ampicillin and growth was monitored by measuring OD\textsubscript{600}. Cells were induced at OD\textsubscript{600} of 2.0-2.5 with 0.1 mM IPTG. Samples were collected at various time points in the post-induction period, cells were pelleted and supernatant was collected as extracellular fraction and assayed for asparaginase activity.

Use of TB supplemented with dextrose did initially result in faster growth of cells and it also appeared to help in reducing of the repression effect on growth due to IPTG induction, thus helping in attaining a higher biomass concentration (Fig 4.34a).

However the extracellular asparaginase yields were higher when glycerol was used as a carbon source (Fig 4.34b). Glycerol is an osmolytic stabilizer and is known to lower the rate of aggregation producing solute interactions (Schein 1990) and glycerol unlike other carbohydrates passes the cell membrane by facilitated diffusion effected by facilitator protein (Lin 1986). Thus it might have the effect of stabilizing proteins inside the periplasm (Lozano et al. 1994).
Effect of temperature on extracellular asparaginase expression

It is now long known that temperature influences the solubility of the recombinant protein being produced. Lowering of growth temperature has also become a successful technique to increase the solubility of expressed proteins in *E. coli*, especially in cases where the protein normally tends to aggregate into inclusion bodies (Schein and Noteborn 1988; Qing et al. 2004). Sub-optimal temperatures (20 - 30°C) of cultivation generally result in increased fraction of soluble form of protein, since protein aggregation increases with increase in temperature and so does the rate of protein synthesis (which in turn increases the inter molecular interactions due to increase in the intracellular recombinant protein concentration). This is a critical factor in determining whether the protein will remain soluble or form misfolded aggregates, as production of the protein in soluble form is considered more of a kinetic phenomenon where the expression rates have to match the rate of folding (Mitraki and King 1989). Also high expression rates increase the concentration of nascent polypeptides in the cytoplasm which results in titration of the foldases (chaperones, prolyl isomerases) that become limiting on overexpression of recombinant proteins (Baneyx and Mujacic 2004).

To check the effect of lowering of growth temperature on extracellular protein yield, PNHAsp cells were grown overnight in a 100 ml flask containing 10 ml LB and used to inoculate two 500 ml flasks carrying 50 ml TB medium each carrying 2X concentration of ampicillin. These culture flasks were grown at 37°C and 30°C respectively. Growth was monitored by measuring OD$_{600}$, and the cells were induced at OD$_{600}$ of 2.0-2.5 with 0.1 mM IPTG. Samples were collected at various time points post-induction, cells were pelleted and supernatant was collected as extracellular fraction and assayed for asparaginase activity. The temperature of 30°C was selected, as it would lower the growth and expression rate slightly without hampering other cellular activities.
As expected the cells grew at a slower at 30°C in comparison to growth at 37°C. Interestingly the cells growing at 30°C did not show the growth repression upon IPTG induction which was typical of growth at 37°C (Fig 4.35 a). This is possibly due to the lowered rates of expression upon induction that didn't unduly stress the cells.

![Figure 4.35 a)](image)

Fig 4.35 a) Growth profile (OD600) and b) Activity profile (Units/ml) of the recombinant PNHAsp cells for extracelluar asparaginase expression over time. Effect of temperature (37°C/30°C) on growth and extracelluar asparaginase expression was analysed.

This is also clear from the product profile where the level of asparaginase secreted to the media was lower throughout the post-induction cultivation period (cultivation at 37°C resulted in ~ 1.3 fold higher final extracellular protein yield than 30°C). However the rate of increase of recombinant asparaginase in the later post-induction period was similar in both the cases (Fig 4.35 b).

Temperature variation has been suggested as a means of influencing the partitioning between the protein inside and outside the cell, since structural changes in the membrane are a function of parameters like temperature (Arneborg et al. 1993). A reduction in temperature reduces the transport (DiRienzo and Inouye 1979), since the membrane goes from a liquid-crystalline bilayer to a more ordered gel phase (van Klompenburg and de Kruijff 1998). Thus temperature is a complex parameter to use specifically for leakage enhancement in this case.

It is clear that cultivation at 37°C leads to higher expression, however this high expression does not lead to recombinant protein build up in the cytosol, since
secretion and excretion rates are also high. Thus the main advantage of cultivation at 30°C (namely reduced expression rates) thus allowing time for proper folding and secretion is not required in this case (confirmed earlier by western blot analysis, see Fig 4.29). However the metabolic stress is definitely reduced and this strategy would be useful for production of toxic proteins or for the expression of proteins which tend to form inclusion bodies where soluble expression is desired.

**Heat-shock based induction strategy for extracellular asparaginase expression**

An alternative induction strategy was followed for production of recombinant asparaginase using a two-plasmid expression system. Here a second plasmid pGP1-2 bearing the kanamycin resistance gene carries the T7 RNA polymerase gene under a heat-shock \( \lambda P_L \) promoter, which is repressed below 30°C but gets induced by heat shock to express T7 RNA polymerase (Tabor and Richardson 1985). There are number of literature reports where the heat shock system has been proven to be a better expression system and very high protein yields have been obtained (Gupta JC et al. 1999; Chao et al. 2002; Menart et al 2003; Srivastava et al. 2005).

![Fig 4.36](image-url) a) Growth profile (OD\(_{600}\)) and b) Activity profile (Units/ml) of the recombinant PNHAsp cells for extracellular asparaginase expression over time. Effect of three heat shock strategies 1) temperature up shift to 37°C, 2) heat shock at 42°C for 2 minutes and 3) heat shock at 42°C for 5 minutes on growth and extracellular expression was analysed.
The assumption here is that transcription is the rate-limiting step. Thus more copies of T7 RNA polymerase and also the stronger $\lambda P_L$ promoter (compared to lac promoter) may improve expression.

Accordingly, BLR (DE3) cells were co-transformed with two plasmids, pPNHAsp and pGP1-2. Cultures were grown overnight in LB with 200 $\mu$g/ml ampicillin (2X) and 50 $\mu$g/ml kanamycin (1X) at 30 °C and this culture was used to inoculate three 500 ml flasks carrying 50 ml TB medium each carrying 2X concentration of ampicillin and 1X kanamycin.

The cultures were grown initially at 30°C to repress product formation. Growth was monitored by measuring OD$_{600}$, and the cells were induced at an OD$_{600}$ of 2.0 - 2.5. In the first case induction was done by shifting the cultures to 37°C, in the second case the culture temperature was up shifted and maintained at 42°C for 2 minutes and then immediately brought down to 37 °C and subsequently grown at 37 °C, while in the third case the temperature up shift was maintained for 5 minutes and then shifted to 37 °C. Samples were collected at various time points post-induction, cells were pelleted and supernatant was collected and assayed for asparaginase activity.

The growth pattern in all the three cases were more or less similar with 5 minutes up shift case showing a marginal suppression of growth in the initial post-induction period (Fig 4.36 a). The activity profiles were quite different with the highest extracellular expression observed in the case of 5 minutes up shift, which however was ~ 1.6 fold lower to that of IPTG induction (middle OD induction cultivated at 37°C). Simple temperature up shifting to 37°C resulted in poor expression and 2 minute up shift gave intermediate values of asparaginase expression levels (Fig 4.36 b). The activity profiles showed a flattening in the rise of activity after 12 hours post-induction in all cases.
Optimization of lactose concentration (inducer) on extracellular asparaginase expression

The natural inducer of the lac promoter, lactose, can also be used as an inducer in recombinant protein expression studies in E. coli where the operator from the 'lac operon' (lacO) is present downstream of the strong promoter (T7, tac etc.). However very few literature reports exist using lactose as an inducer probably due to the greater difficulty in establishing ideal induction conditions since lactose is simultaneously an inducer as well as a carbon source (metabolizable inducer). Thus, the amount of lactose to be used in cultivation needs to be first optimized such that it can be effective as an inducer while simultaneously being used up as a carbon and energy source during cultivation (Donovan et al. 1996; Neubauer and Hofmann 1994; Gombert and Kilikian et al. 1998).

In order to optimize the lactose concentration for obtaining the maximum extracellular protein yield, PNHAsp cells were grown overnight in a 100 ml flask containing 10 ml LB. This was used to inoculate three 500 ml flasks carrying 50 ml TB each carrying 2X concentration of ampicillin and the culture flasks were grown at 37°C.

![Figure 4.37 a) Growth profile (OD₆₀₀) and b) Activity profile (Units/ml) of the recombinant PNHAsp cells for extracellular asparaginase expression over time. Effect of three inducer concentrations of lactose 1) 0.2%, 2) 0.4% and 3) 0.8% on growth and extracellular expression was analysed.](image-url)
Growth was monitored by measuring $OD_{600}$, and the cultures were induced at $OD_{600}$ of 2.0 - 2.5 with varying concentrations of lactose of 0.2%, 0.4% and 0.8% (final concentration). Samples were collected at various time points post-induction, cells were pelleted and supernatant was collected as extracellular fraction and assayed for asparaginase activity.

The growth pattern was similar in all the three cases resulting in similar final biomass yields. However a marked reduction, in the growth repression (which was characteristic of IPTG induction in the initial post-induction period) was observed. Also the use of lactose as an additional carbon source resulted in achieving higher biomass concentration in all cases in comparison to IPTG induction (Fig 4.37 a). The activity profiles also showed a high expression level into the extracellular media, with 0.4% lactose giving the highest asparaginase levels 1.2 fold higher than 0.8% lactose-containing medium. The expression levels achieved here were comparable to IPTG induction (Fig 4.37 b). Thus lactose at optimal concentration of 0.4% was an equally effective inducer at the shake-flask level.

**Effect of additives on extracellular asparaginase expression**

The cell envelope of gram-negative bacteria consists of a cytoplasmic cell membrane and an outer membrane that consists of phospholipids and lipopolysaccharides. Between the outer and cytoplasmic membrane is a thin peptidoglycan layer, forming a support for the cell envelope (Sikkema et al. 1995). The periplasmic space in gram-negative bacteria is the space present in between the plasma membrane and the peptidoglycan layer. This space contains enzymes and proteins functioning in a wide range of processes (Madigan et al. 2003). In order to release these proteins (native/recombinant) from the periplasmic space, the permeability barrier (outer membrane) must be weakened. Chemical methods are readily used to achieve the above-mentioned objective, for this, chemical substances that influence the membrane porosity are used as additives (Naglak and Wang 1990). Here the effect of various additives...
on the permeability of the cellular membrane was analysed in order to improve extracellular expression.

To check the effect of these additives on extracellular asparaginase yield and to optimize the additive concentration, PNHAsp cells were grown overnight in a 100 ml flask containing 10 ml LB and was used to inoculate 500 ml flasks carrying 50 ml TB each carrying 2X concentration of ampicillin. The culture flasks were grown at 37°C, growth was monitored by measuring OD$_{600}$, and the cells were induced at OD$_{600}$ of 2.0-2.5 with 0.1 mM IPTG. Varying concentrations of different additives were added to the growing cultures 2-hours post-induction. A control flask was also grown simultaneously, where no additive was added. Samples were collected at various time points post-induction, cells were pelleted and supernatant was collected and assayed for asparaginase activity.

a) Effect of glycine: Glycine has been found to be able to induce morphological alterations in *E. coli*, such as swelling and elongation, by virtue of the fact that it gets incorporated into precursors of peptidoglycan. This results in the disruption of peptidoglycan cross-linkages and cell membrane integrity (Hammes et al. 1973). Dramatic enhancement of secretion of heterologous proteins into culture media caused by glycine has been reported (Yang et al. 1998; Fujiyama et al. 1995).

Glycine from the sterile stock solution was added 2 hours post-induction in two flasks such that a final concentration of 0.5% and 1% glycine was obtained in the growing culture. One flask was used as control, where culture was induced with 0.1 mM IPTG but glycine was not added.

Addition of glycine had an overall positive influence on the secretion of asparaginase into the culture media. Glycine addition at 1% concentration was more effective in extracellular secretory expression where ~1.3 fold higher volumetric activity was achieved 24 hours post-induction compared to that of the control culture where glycine was not added.
Results & Discussions (shake-flask studies)

Higher glycine concentration (1%) and in the media inhibited the cell growth slightly and also resulted in higher secretory expression per cell as followed by the specific product yield profile (Fig 4.38 a & b).

b) Sodium chloride (NaCl): Addition of salts such as NaCl has been shown to influence the outer membrane structure which in turn can influence the release of periplasmic proteins to the media (McGarrity and Armstrong 1975). Addition of NaCl after induction resulted in an increase in the specific productivity of recombinant protein by three fold (Know et al. 1996).

NaCl from the sterile stock solution was added at 2 hours post-induction in three flasks such that a final concentration of 0.25%, 0.5% and 1% NaCl was obtained in the growing cultures. One flask was used as control where cultures were induced with 0.1 mM IPTG but NaCl was not added.

Increasing NaCl concentration showed a slight negative effect on growth and final cell biomass achieved but had a concentration dependent effect on extracellular asparaginase yield. A ~ 1.2 fold increase in volumetric activity was achieved with 0.25% NaCl concentration in comparison to control.
High concentration of NaCl (1%) influenced the membrane permeability resulting in repression of secretory expression (Fig 4.39a & b).

c) **Triton X-100**: Detergents are commonly used to disrupt the lipid membrane structures for extraction of membrane proteins (Neugebauer 1990). However at low concentrations, detergents partition into the lipid bilayer without causing solubilization (Kragh-Hangen et al. 1993). In low concentrations, Triton X-100 has been shown to exert its major effect on the outer membrane by causing loss of membrane integrity and has a minor effect on the permeability of the inner membranes of *E. coli*. Treatment of native/ recombinant *E. coli* cells have been shown to result in increased protein production by many folds (Yang et al. 1998; Zhao and Yu 2001; Geckil et al. 2005).

Triton X-100 from the sterile stock solution was added at 2 hours post-induction in three flasks such that a final concentration of 0.25%, 0.5% and 1% Triton X-100 was obtained in the growing cultures. One flask was used as control where cultures were induced but Triton X-100 was not added. Addition of Triton X-100 to the culture resulted in attaining higher extracellular asparaginase yields in a concentration dependent manner. Maximum activity was
achieved in case of 1% Triton X-100, which was 1.6 fold higher over the control culture 24 hours post-induction (Fig 4.40 a & b).

The specific product yield was also highest in case of 1% Triton X-100 indicating that this concentration was the most suitable for increasing the membrane permeability. The final biomass obtained was similar in all the cases.

d) Calcium chloride (CaCl₂): Divalent salts are also known to influence the membrane structure of E. coli. Though the mechanism of their action is still not clear, these have been used for release of periplasmic proteins (5 mM MgSO₄), CaCl₂ (100 mM) for making the E. coli membrane competent to plasmid uptake, while MgCl₂ is considered to be a membrane stabilizer (Pugsley and Schwartz 1984). Therefore CaCl₂ was used at lower concentrations to examine its influence on release of recombinant asparaginase to the medium.

CaCl₂ from the sterile stock solution was added at 2 hours post-induction in three flasks such that a final concentration of 5 mM, 10 mM and 25 mM CaCl₂ was obtained in the growing cultures. One flask was used as control where the culture was induced but CaCl₂ was not added.

CaCl₂ at lower concentrations (up to 10 mM) showed a slight positive influence in increasing the asparaginase level in the media. In case of 25 mM CaCl₂ a ~ 1.35
fold lower extracellular asparaginase productivity was obtained compared to the control culture (Fig 4.41 a & b).

![Graph](image)

**Fig 4.41** Effect of CaCl₂ concentration (5 mM, 10 mM and 25 mM) on extracellular asparaginase production. **a)** Volumetric activity/ml and **b)** Specific product yield/100 ml at 24 hour of post-induction cultivation.

Use of 5 mM CaCl₂ resulted in achieving the highest extracellular product concentration (~ 1.2 fold over the control culture). The specific product yield was also highest in case of 5 mM CaCl₂. At its uppermost concentration i.e. 25 mM CaCl₂ inhibited cell growth thus resulting in achieving a low final biomass yield.

e) **Sucrose**: Supplementation of the medium with high concentration of non-metabolizable sugars (such as sucrose or raffinose) has been shown to increase the yields of soluble secreted recombinant proteins in *E. coli* (Bowden and Georgiou 1990). Polyols and sugars presumably prevent the aggregation of the overproduced protein in the periplasm and in-vitro (probably in media as well) these are known to strengthen the water lattice and decrease the partial molar volume of the protein, thus lowering the rate of aggregate producing solute interactions (Lozano et al. 1994; Gekko and Timasheff 1981).

Sucrose from the sterile stock solution was added to cultures 2 hours post-induction in three flasks to a final concentration of 1%, 2% and 3%. One flask was grown as control and induced with 0.1 mM IPTG where sucrose was not added.
Addition of sucrose did have a slight influence on the release of asparaginase into the culture media. However the concentration of sucrose had little effect on the extracellular asparaginase yield. In all three cases more or less similar volumetric activity was obtained, which was ~1.2 fold higher to the control culture (Fig 4.42 a & b).

Use of 3% sucrose resulted in achieving maximum efficiency of secretion per cell with ~1.4 fold higher specific product yield in comparison to the control culture, however 3% sucrose also resulted in reduced levels of final biomass yield in comparison to the other three cultures, thus canceling out the gain obtained by increased specific product yields.

f) Effect of Hexane/EDTA/Toluene/KCl: Hexane, toluene (organic solvents), EDTA (chelating agent) and KCl (salt) are also known to influence the porosity of the outer membrane by causing selective permeabilization of the outer cell wall allowing release of periplasmic enzymes in relatively pure form (Bansal-Mutalik and Gaikar 2003; De Leon et al. 2003). The effect of these additives on the release of recombinant asparaginase was also checked.
Hexane, EDTA, toluene and KCl from the sterile stock solution were added to one flask each to reach a final concentration of 0.4%, 0.5 mM, 0.4% and 0.5% respectively 2 hours post-induction. One flask was used as control where culture was induced with 0.1 mM IPTG but without additive(s).

Among organic solvents hexane was the most effective agent for enzyme release, while the other organic solvent toluene had an antagonistic effect with lowest volumetric yield and specific productivity. Addition of hexane resulted in achieving a ~ 1.3 fold higher volumetric activity and specific product yield over the control culture. KCl did not influence volumetric activity or growth significantly. EDTA on the other hand had an inhibitory effect on the release of asparaginase into the culture media. However EDTA at 0.5 mM concentration did not hamper culture growth (Fig 4.43a & b).

To conclude we did obtain a marginal increase in the extracellular expression with some additives showing a concentration dependent influence on the release of recombinant asparaginase into the media. Triton X-100 was the most effective permeabilization agent with a ~ 1.6 fold increase in volumetric activity and with a specific product yield of ~ 280 Units/OD$_{600}$ over the control culture. On the other hand EDTA and toluene had a negative effect on both the culture growth and volumetric activity.
The additives can be ranked in terms of their effectiveness in a decreasing order as follows:
Triton X-100 > hexane > sucrose > glycine > NaCl > CaCl₂ > KCl > EDTA > toluene.
Attempts were made to use additives in various combinations to obtain significant improvement over these results. However, cellular health was invariably affected by the use of two or more additives in concert, as observed by rapid fall in OD₆₀₀ values.