Chapter 6.

BIOREACTOR STUDIES IN E. COLI & PARTIAL PURIFICATION
After successfully achieving a fairly high specific activity in shake flasks, the next logical step was to translate this into high levels of production in a fermentor. As stated earlier, the idea is to grow cells to a much higher cell density than achievable in shake flask while retaining, to whatever extent possible, their high specific activity. This requires that the cellular health be in no way compromised during the process, which is achieved by a fine tuning of bioprocess parameters. A combination of high activity per unit cell and high biomass per litre of culture then leads to a high level of product.

In our lab we have been working on this problem and have successfully overproduced many recombinant proteins. (Yazdani et al., 1998; Gupta et al., 1999). These range from β-galactosidase to toxic proteins like streptokinase. The basic principles of the overproduction strategy are as follows

- Prevent build up of waste products by using a well-balanced media composition.
- Retain a fairly high specific growth rate of cells, especially at the time of induction, since specific growth rate correlates very well with overall cellular health.
- Prevent acetate accumulation by keeping the concentration of easily assimilable carbon sources (typically glucose) low, since fast consumption of glucose tends to saturate the electron transport chain leading to acetate formation.
- Keep high antibiotic concentration in order to retain selection pressure, especially ampicillin which gets degraded by the β-lactamase secreted by recombinant cells.

Often the above requirements are counterpoised giving a very narrow range of operational flexibility. For example μ values below 0.2 h⁻¹ adversely affect cellular health while values above 0.4 h⁻¹ lead to acetate accumulation. Thus typically fermentation is carried out in three stages. The first is a glycerol batch phase,
which would serve to generate cell mass. The second step is a fed-batch phase where glycerol is added at a growth-controlling rate to reach high cell density. The third is an IPTG induction phase with a fed-batch mode to sustain cell growth while production of the recombinant protein production goes on inside the cells.

6.1 Expression of rhGM-CSF in cells with pRSET-GMCSF

It was decided initially to test the first low producing construct, pRSET-GMCSF, in the fermentor as control. A standard benchtop 2 litre reactor (INFORS AG, CH4103) was set up with the above plan with a 1 litre working volume which was aseptically inoculated with a 100 ml overnight culture of BL21(DE3) cells carrying the plasmid pRSET-GMCSF. The media composition for the batch phase was as follows.

**Media composition (g/l)**

<table>
<thead>
<tr>
<th>Yeast Extract</th>
<th>Tryptone</th>
<th>Glycerol</th>
<th>K$_2$HPO$_4$</th>
<th>KH$_2$PO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>12</td>
<td>5% v/v</td>
<td>12.54</td>
<td>2.31</td>
</tr>
<tr>
<td>Autoclaved in 900ml of H$_2$O in reactor</td>
<td>Autoclaved in 100ml H$_2$O in flask</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Added together just before start of fermentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To enhance plasmid stability a 3X ampicillin concentration was used. The temperature, pH and DO was set at 37°C, 7.2 and 100% respectively with an initial stirrer speed of 200 RPM. The airflow rate was 1 vvm and was not changed during the fermentation. The pH was controlled automatically with dosing pumps using 0.5 N NaOH and 0.5 N HCl. Automatic DO control was set at 40% until the agitation rate reached 1000 rpm. Excess foaming was controlled by adding antifoam (SIGMA).
In the initial stages it was observed that the specific growth rate (μ) of the culture was 0.615 h\(^{-1}\) (Fig. 6.1.). This is in marked contrast to other (non toxic) proteins where typically μ has values of around 1-1.2 h\(^{-1}\). It was earlier observed that pRSET-GMCSF has a leaky background expression which might be one of the causes leading to the inhibition of cell growth even under non-induced conditions. However it should also be noted that our studies with the \(P_{\text{lyS}}\) strain which suppresses leaky expression did not give higher yield. After 5 hours of continuous growth μ started falling and by the end of the 10\(^{th}\) hour it reached a value of 0.047 h\(^{-1}\) which remained constant for the next 4 hours. From the 10\(^{th}\) hour to the end of the 14\(^{th}\) hour no appreciable change in cell density could be observed.
The oxygen uptake rates (OUR) which can be interpreted from the RPM profile clearly show that the trend is radically different than what is observed during conventional recombinant fermentation. Usually the OUR increases with increase in cell density reflecting the increase in growth rate. Here on the contrary with an increase in cell density from 1 to 2.75 gm/l (DCW) it showed a decreasing trend. This could have been because of rhGM-CSF reaching a certain critical level within the growing cells which affects their metabolic machinery quite severely. It is interesting to compare this with the data from the shake flask experiment (Fig.4.10.A,B) where the cells had a pre-induction $\mu$ of 0.47 h$^{-1}$, which changed to a post induction $\mu$ of 0.09 h$^{-1}$. Though the growth rate slowed down as is apparent from the RPM trends and lnOD$_{600}$ data this was not because of any shortage of nutrients since stoichiometric calculation showed that at this biomass level sufficient residual nutrients would remain in the medium. Thus, this slowdown can be attributed to the effect of rhGM-CSF toxicity.

To be doubly sure a pulse feed was given for 5 minutes after 8 hours. No change in RPM, and hence OUR was observed to indicate that it was being utilized. After observing the trend for 13.5 hours, the culture was induced with 1 mM IPTG. The value of $\mu$ fell to 0.025 h$^{-1}$. After 1 hour the volumetric activity of rhGM-CSF reached 59.5 mg/l (as revealed through ELISA) and peaked to a value of 79.34 mg/l of culture broth corresponding to a specific rhGM-CSF concentration of 19 mg/g DCW (1g DCW has about 300mg total protein). This corresponds to about 6.3% of total cellular protein.

At the end of the fermentation (24$^{th}$ hour) 3.54 g/l of DCW was harvested. Hourly samples post induction, which were stored and frozen, were later analyzed on SDS PAGE (Fig. 6.2). RhGM-CSF was visually estimated to represent at least 5% of the total protein of the cell after induction. This matched fairly well with the ELISA data. This level of specific activity corresponds reasonably well with levels obtained at the shake flask studies where the fraction of rhGM-CSF was 6-7% of total cellular protein.
Fig. 6.2. SDS-PAGE showing expression of rhGM-CSF in the fermentor. Lane M: protein molecular weight marker; Lanes 1-6: 1hr, 2hr...6hr after induction.

6.2. Purification of rhGM-CSF using Electro-elution:

In order to compare yields using different purification strategies it was decided to use these fermentor samples where the rhGM-CSF does not contain any affinity tag. This would serve as a control for comparing yields with the affinity tagged protein. Thus cells at the end of the fermentor run were harvested and pelleted. After that they were boiled in sample buffer (in 0.1M Tris/HCl pH 8.0, 1% SDS and 0.1% 2-mercaptoethanol) at 90-100°C for 5 minutes in a water bath and loaded on 15% preparative SDS-PAGE with marker. After CBB-R250 staining, the band of interest was excised from the gel carefully and electroeluted using Biotrap BT-1000™ (Schleicher & Schuell, Germany). For this, the gel fragment was kept in SDS gel running buffer and an electric field applied. The protein
moved out of the gel under this field and was subsequently trapped between two membranes (one having a pore size of 100 kD and the other having a pore size of 1 kD). The eluted protein was collected and extensively dialysed against phosphate buffer (pH 7.3). The protein purified in this way was quantified by ELISA and found to have more than 90% recovery. From the SDS-PAGE we can see that most of the contaminants have been removed. (Fig. 6.3) though the obvious limitation of this protocol is that any contaminating protein co-migrating with rhGM-CSF in SDS-PAGE would remain.

![SDS-PAGE showing coomassie stained band of purified rhGM-CSF after electroelution. Lane M: protein molecular weight marker; Lane P: purified rhGM-CSF.](image)

Fig. 6.3. SDS-PAGE showing coomassie stained band of purified rhGM-CSF after electroelution. Lane M: protein molecular weight marker; Lane P: purified rhGM-CSF.
6.3. Production of rhGM-CSF in fed batch culture with plasmid pET22b-GMCSF:

After studying rhGM-CSF expression in the control it was decided to compare expression in pET22b GMCSF which gave a fairly high level of expression in shake flask cultivation. Like the earlier strategy, a fed batch technique was devised to achieve high cell density. In this experiment, we decided to introduce an exponential feeding strategy in order to maintain the specific growth rate of the cells at a constant value. It has earlier been shown that this strategy allows very little byproduct formation due to the efficient utilization of all the nutrients (Yee et al., 1992). The initial media composition for the batch phase was as follows.

<table>
<thead>
<tr>
<th>Media composition (g/l)</th>
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<tbody>
<tr>
<td>Yeast extract</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>Autoclaved in 900ml of H2O in reactor</td>
</tr>
<tr>
<td>Added together just before start of fermentation</td>
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Media composition (200ml) for feed (30X) was as given below:

<table>
<thead>
<tr>
<th>Media composition (g/l)</th>
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</thead>
<tbody>
<tr>
<td>Yeast extract</td>
</tr>
<tr>
<td>300</td>
</tr>
<tr>
<td>Autoclaved in 500ml of H2O in flask</td>
</tr>
<tr>
<td>Mixed together maintaining the sterility of media</td>
</tr>
</tbody>
</table>

As previously, a 3X ampicillin concentration was used to enhance plasmid stability. The temperature, pH and DO was similarly set at 37°C, 7.2 and 100% respectively. All operational parameters were kept the same as before.
The feed was started when the cells reached an OD_{600} of 8.36. The initial flow rate of the feed was calculated from the following equation,

\[ F = \mu S_o' V / S_0 \]  \hspace{1cm} (i)

Where \( F \) = flow rate of feed (l/h), \( \mu \) = specific growth rate of the cells (h\(^{-1}\)), \( S_o' \) = initial substrate concentration in batch culture (g/l), \( V \) = volume of the reactor (l), \( S_0 \) = substrate concentration in feed (g/l).

The value of \( F \) calculated from the above equation was 9.5 ml/h. The feed was doubled every 2 hours and 20 minutes by incrementally increasing it in steps every 45 min according to the following equation, where the value of \( \mu \) was kept as 0.3 h\(^{-1}\).

\[ F = F_0 e^{\mu t} \]  \hspace{1cm} (ii)

This value of \( \mu \) was sufficiently high to maintain good cellular health before induction and during expression. Simultaneously it helped in maintaining a fairly low glucose concentration of less than 0.1% which in turn prevents acetate build up in the cells. To be doubly sure glucose concentration, was also monitored to keep acetate formation in check. If the increase in feed resulted in an increase in residual glucose concentration above 0.1 %, the previous feed rate was continued to allow glucose concentration to fall. Otherwise the feed rate was increased exponentially. The cell density was measured at regular intervals to ensure that the cells were growing at the required specific growth rate. The culture was induced with 1 mM IPTG at an OD of 22.06.

The specific growth rate was monitored throughout the fermentation and it was maintained close to 0.3 h\(^{-1}\) before induction. The \( \mu \) declined after induction and went as low as 0.048 h\(^{-1}\). The agitation rate was not affected significantly after induction and as soon as the cells recovered from the induction shock, the rpm rose after 3 hrs with \( \mu \) increasing marginally to 0.084 h\(^{-1}\). The profiles of the
Fig. 6.4. Fermentation profiles for the expression of rhGM-CSF in *E. coli* with plasmid pET22b-GMCSF.
various fermentation parameters are shown in figure 6.4. It can be seen from the profile that the feeding rates efficiently controlled the residual glucose concentration in the broth which remained as low as 0.05 % and peaked just once at a relatively low value of 0.1%.

This efficient utilization of glucose helped in avoiding byproduct (acetate) formation as was also evident from the low consumption of base during the fermentation. After 6 hours of induction the cells started growing well again which was observed in the form of an increase in oxygen demand. The specific activity of recombinant hGM-CSF, which was 50mg/g DCW (2 1/2 times the value obtained in the control culture), also peaked around 5-6 hours after induction. This escape from stress and a concomitant drop in expression reflects either plasmid instability (emergence of plasmid free cells in the medium) or a general

Fig. 6.5. SDS-PAGE showing rhGM-CSF production in fermentor using plasmid pET22b-GMCSF. Lanes 1-8: 0hr, 1hr, 2hr.. ....7hr after induction; Lane M: protein molecular weight marker.
decline in the cells capacity to express rhGM-CSF and hence a decline in the metabolic load on the cells. The maximum volumetric productivity could be seen at around 8 hours after induction to a fairly high value of about 900 mg per litre of culture broth. This represents a 12 fold increase over the values obtained in the control culture.

The samples of each hour after induction were analyzed on SDS-PAGE (Fig 6.5). The SDS-PAGE data are fairly consistent with the ELISA data that the specific activity of rhGM-CSF peaks during the 5-6th hour following induction.

6.4. Purification of rhGM-CSF after HCDC using affinity chromatography:

To compare purification yields with the earlier control, fermenter samples were collected and rhGM-CSF purified from the cell lysates using Ni-NTA affinity chromatography. 100 ml of the culture was taken, the cells pelleted and

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**Fig. 6.6.** SDS-PAGE showing purification of 6-His tagged rhGM-CSF with metal affinity chromatography (Ni column). Lane M: protein molecular weight marker; Lane 1: whole cell lysate; Lane 2: urea treated lysate; Lane 3: unbound proteins after wash; lanes 4,5,...,9: elution profile (1ml each) using 250 mM imidazole.

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lysed and the denatured cell lysate loaded on to the column denaturing conditions. The rhGM-CSF protein contains the 6-histidine tag at its N-terminus. These histidines interact and bind with the Ni ions of the Ni-NTA matrix. Stringent washing conditions (30 mM imidazole) were used to reduce non-specific binding and get fairly pure protein in a simple purification step (Fig. 6.6). Elution was done at 250 mM imidazole.

The protein after affinity chromatography contained the full-length rhGM-CSF. The protein yields of 460 mg/l were obtained which translates to about 50% recovery.

6.5. Production of MBP-GMCSF fusion protein in fed batch culture:

Finally the MBP-rhGMCSF fusion was tested in a bioreactor since it gave the highest specific activity in shake flask culture. The initial media composition for the batch and fed batch phase was the same as used previously (i.e. with plasmid pET22b-GMCSF). The temperature, pH and DO was set at 37°C, 7.2 and 100% respectively as before. Initial stirrer speed, airflow, pH control and automatic DO control was set as with the fermentor run with pET22b-GMCSF. Antifoam was used occasionally as required.

The fed-batch phase was initiated at an OD_{600} of 5.0 with a feed rate of 6 ml/h. The flow rate of the feed was calculated from equation described in section 7.1.1 by equations (i) and (ii). The entire fed-batch phase was carried out under glucose-limiting conditions. Steady build-up of biomass (upto 9.2 g/l) was accomplished during fed-batch before induction. Feed was increased exponentially, doubling every hour, to get a $\mu$ of 0.693 h^{-1} (since we got a high $\mu$ of 0.89 in the batch phase) but this sp growth rate could not be sustained at higher OD values and thus $\mu$ was only 0.57. Correspondingly a very slight increase in glucose build up was observed since all the feed was not consumed.
Fig. 6.7. Fermentation profiles for the expression of MBP-GMCSF fusion protein in E. coli.
After IPTG induction, $\mu$ declined moderately to a value of 0.252 h$^{-1}$ due to the metabolic burden of recombinant protein production. The residual glucose concentration had a maximum value of 0.046% and thus byproduct (acetate) formation was mostly avoided. The oxygen requirement increased steadily after induction indicating that the growth rate was not affected significantly. This, and comparatively small decline in $\mu$ values post induction indicate that recombinant toxicity was much lower than that observed in the case of pET22b-GMCSF fermentation where the RPM value reached a plateau after induction and the $\mu$ declined to 0.048 h$^{-1}$.

The volumetric concentration of the recombinant protein reached 1.7 g/l of culture broth, corresponding to a specific product concentration of 50 mg/g DCW after 4-5 hours of induction. This compares favourably with the specific activities in shake flask culture where the product concentration was 48.2 mg/g DCW. Thus the fermentation strategy was able to maintain the cellular health of cell while increasing the biomass concentration 20 times. This increase in biomass helped increase volumetric productivity 24.4 fold to 1.7g/l. The majority of GMCSF was produced during the first 5 hours of IPTG induction. Thereafter, the volumetric concentration of the recombinant product did not increase significantly despite the continued addition of the feed medium. Along with the specific productivity reaching a plateau about 5-6 hours after induction, a retardation of cell density as well as volumetric productivity was also observed. The profiles of residual glucose, feed rate and oxygen requirement are shown in figure 6.7.

Figure 6.8 shows the SDS-PAGE analysis of total cell lysates prepared after IPTG induction where the MBP-rhGMCSF band is ~15% of the total protein. From the ELISA data the fraction of MBP-rhGMCSF fusion should correspond to 20% of total protein thus matching the SDS-PAGE data.
Fig. 6.8. SDS-PAGE showing MBP-rhGMCSF fusion protein production in the fermentor. Lane M: protein molecular weight marker; Lane 1-6: 1hr, 2hr, 6hr after induction.

6.6. Purification of rhGM-CSF after HCDC using affinity chromatography:

The rhGM-CSF was purified under native conditions using an amylose column (NEB) in order to compare this purification strategy with two previous ones. The expressed proteins bear maltose binding protein (MBP) fused to their N-termini. MBP interacts and binds with the amylose of the matrix immobilized in the column. The cell lysate was passed through an amylose column with 5 ml resin. The column was washed to remove non-specific bindings. Finally the column was washed with a buffer containing excess maltose, resulting in the elution of the purified MBP-rhGMCSF protein (Fig. 6.9). This purified fusion protein was digested with factor Xa which cleaved the fusion into two fragments, MBP and rhGM-CSF. RhGM-CSF was purified from this mixture using size exclusion chromatography (Sephadex G-25).
Fig. 6.9. SDS-PAGE showing Factor Xa digestion of fusion protein purified from amylose column. Lane M: protein molecular weight marker; Lane 1: whole cell lysate of fermentation sample; Lane 2: MBP-GMCSF fusion protein eluted from amylose column; Lane 3: Factor Xa digested fusion protein releasing MBP and rhGM-CSF.

The protein after affinity chromatography and size exclusion contained the full-length rhGM-CSF. A final yield of 175 mg/l of culture broth of rhGM-CSF was obtained after purification.

This corresponds to,

\[(0.175 / 1.7) \times (58 / 15) = 39.8\% \text{ recovery}\]

6.7. Discussions:

The fermentation strategies helped us to reach close to gram level productions per litre of rhGM-CSF. This is by far the highest amounts of rhGM-CSF expression reported in literature. Of course, rhGM-CSF being a commercially
important protein, most of the expression data would be held by big pharmaceutical companies and not be available in the public domain. This underlines the importance of these results since they can be used to develop indigenous technologies for production. The highest yield obtained was for the MBP-rhGMCSF fusion (1.7g/l) primarily because MBP masks the toxic effects of the rhGM-CSF protein. Unfortunately a large fraction of this protein consists of the MBP which in turn lessens the final yield of rhGM-CSF which is higher in the 6-His tagged system. Thus from the point of view of developing technologies for production, the 6-His tag system scores over the others in terms of maximum final yields. Of course the final cost calculations need to take into account whether the His tag needs to be removed prior to drug formulation.

From the bioprocess perspective the fermentation strategies were a success since they were able to retain (and improve) the specific activities obtained in shake flasks thus volumetric productivities increased proportionally to biomass levels. Though the final OD's obtained were fairly high (50) there are reports of HCDC achieving even higher values without compromising the specific activities. This exercise was not undertaken due to lack of time. Rather it was decided to study alternative hosts, namely Pichia pastoris and Pichia methanloica where high cell densities are routinely achievable in fed batch culture.

However before this could be undertaken it was necessary to test for the biological activity of the product since misfolding and correct disulphide bond formation is quite common in E. coli especially while expressing human proteins.