CHAPTER 5.

BIOREACTOR STUDIES OF hGM-CSF EXPRESSION in E. coli
5. Bioreactor studies of hGM-CSF expression in *E. coli*

5.1 Production of hGM-CSF in batch culture

Unlike shake flask cultures, a bioreactor provides better control over bioprocess parameters like pH and dissolved oxygen concentration. Hence batch cultivation was initially done to test the level of recombinant hGM-CSF expression in a bioreactor. The temperature, pH and dissolved oxygen concentration (DO) was set at 37 °C, 7.2 and 40 % respectively with an initial stirrer speed of 250 RPM. The DO concentration was maintained by feed back control of the RPM when it fell to 40%. The pH was controlled using 1 N NaOH and 1N HCl using a PID controller. The airflow rate was maintained at a constant value of 1 vvm through out the cultivation. Glycerol was replaced with glucose as a carbon source because there are fast and reliable assay techniques available for monitoring glucose levels off-line. Thus, the bioreactor studies were conducted in a slightly different media from what was used earlier in shake flasks, though a number of experiments carried out in shake flasks had shown that the effects of change in carbon source (from glycerol to glucose) was not very significant.

BLR (DE3) *E. coli* cells transformed with the pETA-GM recombinant plasmid were inoculated in 15 ml TB glucose medium having 50 µg/ml of kanamycin. Cells were grown overnight at 37 °C with constant shaking at 250 RPM. 5 ml of this overnight grown culture was inoculated in 100 ml TB glucose with kanamycin and grown till a cell density (OD 600) of ~3. This secondary culture was used to inoculate the bioreactor having 1L TB glucose medium containing 75 µg/ml of kanamycin. Cells were grown till a OD 600 of 10 after which they were induced by 0.5 mM IPTG. Samples were taken at regular time intervals of 1 hour, post induction.

From the growth profile a continuous decline in the biomass concentration (OD 600) was observed after 1 hour of induction (Fig 5.1a). A maximum biomass concentration (OD 600) of 12.2 was achieved which finally declined to 10.2 in 6 hours post induction. The specific growth (µ) fell from 0.54 h⁻¹ pre-induction to 0.18 h⁻¹ within the 1st hour of induction. Since batch runs with TB medium of un-induced cultures gave a final OD 600 of 20-22 (data not shown), it can be safely assumed that substrate availability was not the limiting factor for cell growth. It is possible that the stress associated with recombinant
protein expression was the reason behind this growth retardation. Even though no growth was observed after 1 hour post-induction, the metabolic activity of the cells did not stop immediately rather it declined gradually as was evident from the gradual fall in RPM, even after stoppage of growth (Fig 5.1a) {RPM was an useful on-line indicator of the oxygen transfer rate (OTR), which matches the oxygen uptake rate (OUR) when DO is at steady state}. The OUR in turn can be correlated with the metabolic activity of the culture.

**Figure 5.1-** a). Time profile of growth (OD$_{600}$), pH and RPM in batch cultivation of *E. coli* BLR DE3 cells having pETA-GM recombinant plasmid. b). Plot showing growth (lnOD$_{600}$) and volumetric product concentration of hGM-CSF in *E. coli* batch culture (induction at OD$_{600}$ of 10).
ELISA was done to quantify the hGM-CSF concentration. From the product profile a continuous increase in volumetric product concentration of hGM-CSF was observed till 5 hours post induction to reach a maximum value of 560 mg/l (Fig 5.1b). A similar trend was also observed in the specific product yield ($Y_{P/X}$) of hGM-CSF which reached a maximum of 137 mg/g DCW after 6 hours post induction (Fig 5.2a). To confirm the above results SDS PAGE of the whole cell lysate was done. A continuous increase in the band intensity of hGM-CSF was observed till 5 hours confirming the increase in buildup of product inside the cell (Fig 5.2b).

The growth and product profile was thus similar to the shake flask results, where also a specific product yield ($Y_{P/X}$) (137 mg/g DCW) had been obtained. However the volumetric product yield increased 1.6 folds due to the higher levels of final biomass obtained in the fermentor.

The initial specific product formation rate ($q_p$) at the time of induction was 42 mg/g DCW/h which declined to zero within 6 hours demonstrating that the ability of the cells to produce hGM-CSF declined with time (Fig 5.3c). Though the measurement of residual substrate concentration was problematic (given the presence of complex substrates in the media) an indirect measure of the metabolic activity could be obtained from the RPM and dissolved oxygen profile which gave us a measure of the oxygen uptake rate (OUR).
continuous decrease in OUR demonstrated the slow drop of culture viability post induction. This loss in cell viability is one of the primary reasons behind the fall in specific product formation rate ($q_p$).

![Plot showing the specific product formation rate ($q_p$) in batch fermentation (induction at OD$_{600}$ of 10).]

**Fig 5.2c**- Plot showing the specific product formation rate ($q_p$) in batch fermentation (induction at OD$_{600}$ of 10).

In order to better understand this kinetics of growth and product formation, continuous culture studies were conducted at different dilution rates ($D$).
5.2 Continuous cultivation studies

Continuous culture studies are a valuable tool for the determination of process kinetic parameters like: biomass yield ($Y_{X/S}$), product yield ($Y_{P/X}$), specific product formation rate ($q_p$), specific growth rate ($\mu$) and maintenance coefficient ($m$) and the interrelationship between these parameters. The expression of intracellular recombinant proteins is critically influenced by the specific growth rate both pre and post induction and the changes in cellular physiology which influence the carbon supply for biomass synthesis (Stephanopoulos and Vallino 1991; Turner et al., 1994). An understanding of the underlying physiology of the organism can provide interesting insights for the design of a bioprocess strategy as well as the basis of model-based control of fed-batch cultivations for the production of recombinant proteins (Schugerl 2001).

*E. coli* is considered to be a preferred host for the scale up of recombinant protein production. However there is a persisting problem of acetic acid formation which in turn hampers both cell growth as well as product formation rates in high cell density fed-batch cultures (Lee 1996; Holms 1996). This is also true when continuous culture techniques are applied (Curless et al., 1994). The induction of recombinant protein synthesis strongly inhibits the specific uptake capacities for substrate and oxygen of the host cells (Neubauer et al., 2003).

To estimate the kinetics of growth and product formation as a function of culture conditions, a continuous cultivation strategy was adopted by running the bioreactor at different dilution rates ($D = F/V$). In this technique, we can control the preinduction specific growth rate. There are reports where a strong correlation between $\mu$ and $q_p$ has been observed in recombinant *E. coli* cultures. There are other reports demonstrating the importance of preinduction $\mu$ which need to be high in order to obtain high levels of recombinant protein expression, this in turn directly correlates with the better cellular health (Siegal and Rue 1985; Jung et al., 1988). However in a few reports, a decline in recombinant protein expression was shown when preinduction $\mu$ was increased (Turner et al., 1994; Seo and Bailey 1986). Finally for some cases no correlation was observed between specific growth rate ($\mu$) and specific product formation rates ($q_p$) (Shin et al., 1997; Jensen and Pederson 1990). Thus the correlation between $\mu$ and $q_p$ seems empirical.
and specific for each system depending on the nature of the recombinant protein, promoter and/or host vector combinations. There is no report of a systematic study of the kinetics of hGM-CSF expression in \textit{E. coli}. This could be because of the extremely poor expression levels obtained by most researchers (Bhattacharya et al., 2005; Berges et al., 1996). So it was decided to study the expression of hGM-CSF in continuous cultivation to obtain the kinetic parameters which in turn would help in designing the bioprocess strategies for high cell density fed-batch cultivation.

The CSTR studies were conducted in TB glucose medium in a 1L bioreactor having pH and DO control (in cascade with RPM and gas mixing). \textit{E. coli} BLR(DE3) cells containing pETA-GM were inoculated in 15 ml TB glucose medium containing 1.5X (75 µg/ml) kanamycin and grown at 37 °C with constant shaking at 250 rpm for 12 hours. 5 ml of this overnight grown culture was further inoculated in 100 ml TB glucose medium containing kanamycin and grown till a cell OD$_{600}$ of ~ 4; this was the seed culture for the bioreactor. This seed culture was used to inoculate a bioreactor containing 900 ml of TB glucose medium having 1.5X of kanamycin. The cells were grown in batch mode till a cell density (OD$_{600}$) of 10 was obtained (mid-log phase) after which a feed was attached at the predetermined dilution rate. Simultaneously the outflow was started and the system allowed to reach steady state before induction with IPTG (0.5 mM). The cultivation studies were conducted at pH 7.3 and 37 °C with a constant air flow rate of 1 vvm. The dissolved oxygen concentration (DO) was controlled at 40%.

\textbf{5.2.1 Continuous cultivation studies at a dilution rate of 0.2 h$^{-1}$}

Cells were grown in TB glucose medium till a cell biomass (OD$_{600}$) of 10. The feed was started at a dilution rate of D = 0.2h$^{-1}$ (200 ml/h) and approximately three reactor volumes were allowed to flow through to obtain a steady state which could be checked by constant biomass concentration (OD$_{600}$), RPM, residual glucose concentration and the DO value. The culture was induced with 0.5 mM IPTG at the steady state biomass concentration (OD$_{600}$) of 16.5. The growth and product profile was monitored continuously post induction. The biomass concentration declined continuously from the steady state value of 16.5 (OD$_{600}$) to 6.9 in 8 hours post induction. The post induction specific growth rate
(μ) which could be derived from the slope of the lnOD₆₀₀ vs time plot (Fig 5.3a) fell from 0.2 h⁻¹ to a constant value of 0.09 h⁻¹ within an hour of induction. This fall in specific growth rate post induction is a reflection of the metabolic burden associated with the expression of hGM-CSF. However the product concentration increased continuously to a maximum hGM-CSF concentration of 346 mg/l, which was obtained 6 hours post induction, after which it declined to 250 mg/l (Fig 5.3a).

However this decline in product concentration was primarily due to the slow washout of cells from the CSTR and not due to proteolytic degradation as is clear from the profile of the specific product concentration (Yₚₓ) which increased linearly till 6 hours of induction after which it tapered off to a value of 87.5 mg/g DCW (Fig 5.3b). The plasmid stability of pre and post induction samples was also checked and it was observed that the culture remained stable throughout the cultivation period.

5.2.2 Continuous cultivation studies at dilution rate of 0.3 h⁻¹

At a higher dilution rate of 0.3 h⁻¹ a steady state biomass concentration (OD₆₀₀) of 15.2 was achieved and cells were induced with 0.5 mM IPTG. The biomass concentration (OD₆₀₀) declined continuously from 15.2 to 4.5 in 5.5 hours post induction (Fig 5.4a),
giving a post induction specific growth rate ($\mu$) of 0.138 h$^{-1}$. This higher value of $\mu$ post induction demonstrates the positive effect of pre-induction $\mu$ on cellular health and thus an improved capacity to handle the metabolic stress associated with recombinant protein production. From the product profile a continuous buildup of hGM-CSF was observed till 3.5 hours of induction, where it reached a maximum of 474 mg/l after which it started declining primarily because of the slow wash out of cells post induction (Fig 5.4a).

![Figure 5.4](image)

**Figure 5.4** - Continuous cultivation studies for the production of hGM-CSF in BLR(DE3) *E. coli* cells at a dilution rate of $D = 0.3$ h$^{-1}$.

- **a)** Growth and product profile.
- **b)** Specific product yield ($Y_{P/X}$) of hGM-CSF

The specific product yield ($Y_{P/X}$) which represents the buildup of the protein inside the individual cell increased continuously till 4.5 hours of induction after which it became constant. A maximum specific product yield of 138 mg/g DCW was obtained representing around 30 % of the total cellular protein which was 50 % higher than the previous result (Fig 5.4b). The cultivation pH declined continuously post induction from 7.3 to 7.08 unlike the previous run at lower dilution rate where it had remained constant and simultaneously an increase in residual glucose concentration was observed after 1.5 h of induction. This decline in pH could be because of the accumulation of acetic acid triggered by the increased levels of glucose in the culture medium.
5.2.3 Continuous cultivation studies at dilution rate of 0.4 h⁻¹

Though we observed a slow washout at a dilution rate of 0.3 h⁻¹, an increased volumetric as well as specific product concentration was obtained primarily because of the positive effect of a higher pre-induction specific growth rate. It was therefore decided to test the

![Graph](image)

**Figure 5.5-** Continuous cultivation studies for the production of hGM-CSF in BLR(DE3) *E. coli* cells at a dilution rate of D = 0.4 h⁻¹.

a) Growth and product profile.

b) Specific product yield (Yₚₓ) for hGM-CSF

expression at a higher dilution rate of 0.4 h⁻¹. A steady state biomass concentration (OD₆₀₀) of 14.6 was obtained at which point the culture was induced by 0.5 mM IPTG. A continuous decline in biomass concentration (OD₆₀₀) from 14.6 to 4.3 was observed within 5 hours post induction indicating that the cells grew at a specific growth rate of 0.178 h⁻¹ in this period (Fig 5.5a). There was a rise in the residual glucose concentration accompanied by a sharp decline in pH indicating an accumulation of acetic acid possibly because of glucose overflow metabolism (Rothen et al., 1998; Xu et al., 1999). The volumetric product concentration increased till 3 hours of induction to reach 276 mg/l after which it declined sharply (Fig 5.5a). This decline in volumetric product concentration was primarily because of the washout of cells at this higher dilution rate. However the specific product concentration (Yₚₓ) increased till 3 hours of induction after which it tapered off to reach a maximum of 82 mg/g DCW (Fig 5.5b).
5.2.4 Discussion

The most efficient glucose utilization took place at a low dilution rate of 0.2 h\(^{-1}\) and the glucose level rose marginally only after 6 hours post induction (Fig 5.6b). Thus the pH also did not change during the post induction period (Fig 5.6a). However at higher dilution rates of 0.3 h\(^{-1}\) and 0.4 h\(^{-1}\), the glucose levels started rising within 2 hours post induction. This clearly shows that the stress associated with recombinant protein expression not only reduced the specific growth rate but also tended to reduce the ability of the cell to utilize substrate (Neubauer et al., 2003). Simultaneously a drop in pH was observed indicating glucose overflow metabolism (Han et al., 1992; Dittrich et al., 2005). This observation is similar to earlier reports that have shown that there is an imbalance created between flux rates of the TCA and Krebs cycle because TCA cycle intermediates are utilized for recombinant protein production (Han et al., 1992). This leads to buildup of pyruvate which in turns gets converted to acetate (Dittrich et al., 2005). The fall in pH was most significant at D = 0.4 h\(^{-1}\) where base addition had to be done after 3 hours when the pH fell below 6.8. Thus acetate accumulation was the

![Figure 5.6- Continuous cultivation studies for the production of hGM-CSF in BLR (DE3) E. coli cells at different dilution rates (D) of 0.2 h\(^{-1}\), 0.3 h\(^{-1}\) and 0.4 h\(^{-1}\).

a) pH profile at different dilution rates post induction.

b) Residual glucose concentration at different dilution rates post induction.](image-url)
probable reason for the lower specific product formation rates \((q_p)\) observed at this dilution rate, since it is well established that acetate interferes with recombinant protein expression (Luli and Strohl 1990; Dittrich et al., 2005).

The rate of protein build up inside the cell (measured by \(Y_{P/X}\)) is determined by the relative rates of growth and product formation and thus it is difficult to interpret this data meaningfully and correlate it with the physiological condition of the cells in the culture. Specific product formation rates \((q_p)\) were therefore calculated by a material balance on the product and biomass (see Box 1). The maximum specific product formation rates \((q_p)\) were observed at an intermediate dilution rate of \(0.3\, \text{h}^{-1}\) (Fig 5.7). A lower dilution rate led to poorer cellular health while a higher dilution rate created the problem of acetate accumulation leading to the existence of an optimal dilution rate for maximal expression (Van de walle and Shiloach 1998). It was interesting to note that at low dilution rates the \(q_p\) value could be sustained for the longest time period of 6 hours clearly indicating that the stress associated with lower levels of protein expression did not impact on the expression capacity of the cell. This aspect is also clear from the glucose and pH profile at the lower dilution rate (Fig 5.6a, b). The results also demonstrate that the buildup of recombinant protein inside the cell does not feed back regulate its own expression. A possible reason is that the recombinant hGM-CSF is present mostly in inclusion body form inside the cell and is thus not toxic to the host. This is in contrast to soluble hGM-CSF expression where the cellular health gets drastically affected by recombinant protein expression (Berges et al., 1996).

As opposed to this low but sustained expression at a lower dilution rate of \(0.2\, \text{h}^{-1}\), the specific product formation rate was initially four folds higher when the dilution rate was \(0.3\, \text{h}^{-1}\). This specific product formation rate however declined rapidly to reach values similar to that observed for the lower dilution rate within 6 hours post induction. This is

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**Box 1**

**Material balance on product and biomass**

\[ Y_{P/X} = P/X \]

Differentiating the above equation, we got

\[
d(Y_{P/X})/dt = XdP/dt - PdX/dt \]

\[= 1/X (dP/dt) - P/X \{(1/X)dX/dt\} \]

\[= q_p - Y_{P/X}\mu \text{ (where } \mu = \{(1/X)dX/dt\} \]

\[q_p = d(Y_{P/X})/dt + Y_{P/X}\mu \]

\(\mu\) could be calculated from the lnOD\(_{600}\) vs time plot
clearly because the fluxes involved in recombinant protein expression rapidly deplete the concentration of critical internal metabolites which can not be replenished by the cell during the post induction period of growth.

\[ \text{Figure 5.7- A comparative analysis of specific product formation rate (} q_p \text{) of hGM-CSF production in } E. \text{ coli at different dilution rates (} D= 0.2 \text{ h}^{-1}, 0.3 \text{ h}^{-1} \text{ and } 0.4 \text{ h}^{-1}). \]

It was expected that at an even higher dilution rate of 0.4 h\(^{-1}\) we would get a further increase in the specific product formation rate (\(q_p\)). However the value of \(q_p\) was consistently lower primarily because of the deleterious effects of acetate accumulation.

An interesting aspect of these experiments was the change in the biomass yield coefficient (\(Y_{X/S}\)) pre and post induction. It is of course extremely problematic to define a biomass yield with respect to glucose given that a complex medium also contains alternative carbon sources. However given these limitations, if we use the measured values of the residual glucose concentration at \(D= 0.2 \text{ h}^{-1}\) for the first 6 hours, we can write the material balance on substrate (see Box 2).

However the \(\ln \text{OD}_{600}\) vs time profile showed that \(\mu\) post induction had fallen to 0.09 h\(^{-1}\) from 0.2 h\(^{-1}\) preinduction. A simple calculation shows that the biomass yield coefficient (\(Y_{X/S}\)) fell to less than half of its preinduction values. Thus there is major increase in the maintenance requirement post induction similar to the observations made by earlier authors (Bhattacharya and Dubey 1997; Wong et al., 1998). We performed similar calculations for higher dilution rates; however since both substrate and biomass concentrations changed significantly post induction (unsteady state CSTR) there was a
need to solve the differential equation for substrate balance. The statistical variation in the data did not allow us to compute the changes in $Y_{X/S}$ values with reasonable precision. However as a conservative estimate, the $Y_{X/S}$ value post induction fell to less than 50% of the preinduction value. This is to be expected since the level of recombinant protein expression was higher at higher dilution rates. A conservative estimate of maintenance coefficient from data obtained from the lower dilution rate of 0.2 h$^{-1}$ was 0.1 h$^{-1}$ (g/g DCW). Even though this data must be treated with caution because of the presence of complex carbon sources in the culture medium, it was useful in the setting up of substrate balances in fed-batch culture (since the media composition was essentially similar).

### Box 2

The substrate balance in a CSTR:

$$\frac{dS}{dt} = D (S_p - S) - \mu x Y_{X/S}$$  \hspace{1cm} (1)

We clearly see no accumulation of glucose and hence $dS/dt$ can be set equal to zero ($dS/dt = 0$)

$$D (S_p - S) = \mu x Y_{X/S}$$  \hspace{1cm} (2)

Preinduction:

$\mu = D$ hence equation 2 can be written as

$$(S_p - S) = x/Y_{X/S}^{Pre}$$  \hspace{1cm} (3)

Post induction:

$$0.2(S_p - S) = 0.09 x/Y_{X/S}^{Post}$$  \hspace{1cm} (4)

Dividing equation 3 by equation 4 We get

$$Y_{X/S}^{Pre} = \sim 2.2 Y_{X/S}^{Post}$$

Assuming that $x$ is a constant. Since $x$ values fall slightly post induction, $Y_{X/S}^{Post}$ is even lower.

#### 5.3 Fed-batch cultivation studies

*E. coli* has been the workhorse for the expression and scale up of recombinant protein production for many years. Fed-batch culture has been used most often to achieve high cell densities and thereby improves the volumetric productivity of the desired protein (Kleman and Strohl 1994). However in most cases of high cell density fermentation, maximizing the cell concentration has helped in increasing the volumetric product concentration only at the cost of lower specific product concentration ($Y_{PX}$) (Kleman and Strohl 1994).

The preinduction specific growth rate is known to be an important factor that affects the production yields of recombinant proteins in *E. coli* (Curless et al., 1990, 1994). A high pre-induction specific growth rate helps in maintaining optimum cellular health which is...
a primary requirement to overcome the metabolic stress associated with recombinant protein production (Sanden et al., 2003). However it is very difficult to control post induction specific growth which often becomes an intrinsic property of the host cell, post induction (Panda et al., 1999; Wong et al., 1998). It has also been shown that the biomass concentration at the time of induction can have considerable effects on the final expression yields achieved (Jeong and Lee 1999; Kwon et al., 1996).

As a consequence of the metabolic burden imposed due to recombinant protein expression, the biochemical and physiological characteristics of the host may be dramatically altered because of diversion of resources away from the metabolism of the host cells. Furthermore, some foreign proteins may interfere with host cell functioning or turn out to be toxic to the host cells (Diaz Ricci and Hernandez 2000). When a foreign protein is over-expressed and exported from the cytoplasm to either the periplasmic space or growth medium, it is possible for the recombinant protein to jam the export sites and prevent the proper localization of essential proteins, which leads to the phenomenon known as “overproduction lethality” (Andersson et al., 1996; Nystrom 2003). The development of a non-dividing population with some degree of metabolic activity after overexpression of a recombinant gene is sometimes comparable to the status of viable but non culturable cells (VBNC) (Nystrom 2003). Due to the hyper expression of a foreign gene, the physiological functions of the host cell are severely affected due to the competition for essential metabolite between recombinant product synthesis and the formation of cellular housekeeping proteins (Diaz Ricci and Hernadez 2000; Corchero and Villaverde 1998). The production of toxic proteins tends to reduce, or even in some cases stop the cell growth post induction (Otto et al., 1995; Asoh et al., 1998). The nature of stress when recombinant protein expression is in the form of inclusion bodies (Jurgen et al., 2000) is however quite different from that of expression in soluble form (Schweder et al., 2002).

The availability of an exogenous supply of essential nutrients like amino acids is also a critical factor in recombinant protein expression (Yee and Blanch 1992; Illanes 1993). Therefore, designing appropriate fed-batch strategies and a proper feed medium plays an important role in final productivities (Niccolai et al., 2003).
It was observed in the previous continuous cultivation studies that the maximum specific product concentration ($Y_{P/x}$) and specific production formation rate ($q_p$) was achieved at a dilution rate of 0.3 h$^{-1}$. A specific growth rate of 0.3 h$^{-1}$ is considered high enough to maintain a good cellular health and at the same time low enough to prevent acetate accumulation especially when complex nitrogen sources are fed along with glucose. Cultivation at a lower specific growth rate often results in changes in the shape of the host cells which become small and round, and these in turn influence the production abilities (Prytz et al., 2003) whereas a high specific growth rate often leads to acetate accumulation (Holms 1986). Hence it was decided to conduct the fed-batch cultivation at the previously determined optimal specific growth rate ($\mu$) of 0.3 h$^{-1}$.

5.3.1 Fed batch cultivation with induction at a cell density of 60 (OD$_{600}$)

Fed-batch studies were carried out in an Infors AG computer controlled bioreactor of 2L with a working volume of 1litre. The batch medium contained TB glucose medium having 75 $\mu$g/ml of kanamycin. The other cultivation conditions were similar to the earlier batch run. BLR (DE3) E. coli cells containing pETA-GM was inoculated in 15 ml TB glucose medium having kanamycin and grown at 37 °C overnight with constant shaking of 250 RPM. 5 ml of this overnight grown culture was further inoculated in 100 ml TB glucose medium having 75 $\mu$g/ml of kanamycin. This secondary inoculum (seed culture) was grown till an OD$_{600}$ of ~3 and the fermentor was inoculated with this seed culture. The cells were grown in batch mode till a biomass concentration (OD$_{600}$) of 14, after which a concentrated feed solution (9% yeast extract, 9% tryptone, 13.5% glucose) was attached at a rate of 15 ml/h. This feed was increased exponentially to maintain a specific growth rate of 0.3 h$^{-1}$ by using a variable speed peristaltic pump. The feed was increased by using following equation:

$$F = F_0 e^{\mu t}$$

Where $F_0$ is the initial flow rate, $F$ is the flow rate at any given time, $\mu$ is specific growth rate and $t$ is time in hours. The feed was thus doubled every 2 hours and 20 minutes by
increasing it in steps every 40 minutes according to the above equation, where the value of specific growth rate was kept at 0.3 h\(^{-1}\).

The culture was induced at an OD\(_{600}\) of 60 with 0.5 mM IPTG. From the growth profile a pre-induction \(\mu\) of 0.27 h\(^{-1}\) was obtained which was constant till the point of induction (Fig 5.8a). A maximum OD\(_{600}\) of 67 was achieved with in an hour of induction which implies that the cells grew at an average \(\mu\) of 0.11 h\(^{-1}\) during this period. No further growth was observed and the OD\(_{600}\) declined to a final value of 58 after 6 hours. The substrate uptake rate fell continuously in the post induction period and thus the post induction feeding rate was also reduced exponentially so as to control the residual glucose concentration around 100 mg/dl. The gas mixing was also decreased to maintain a constant dissolved oxygen concentration of 40%. The decrease in the metabolic activity of the cells post induction was reflected in the decrease in feed rate as well as OUR (Fig 5.8a).

![Figure 5.8- a). Time profile of growth (OD\(_{600}\)), feed rate, pH and residual glucose concentration in fed-batch cultivation of E. coli cells (induction at OD\(_{600}\) of 60).

b). Plot showing growth (lnOD\(_{600}\)) and volumetric product concentration of hGM-CSF in E. coli fed-batch culture (induction at OD\(_{600}\) of 60).]
From the product profile a continuous increase in volumetric product concentration of hGM-CSF was observed. A maximum product concentration of 2.75 g/l was obtained after 5 hours post induction, which did not increase further (Fig 5.8b). The specific product yield \( Y_{P/X} \) also showed a similar pattern since there was very little change in the biomass concentration and a maximum value of 115 mg/g DCW of hGM-CSF was obtained after 6 hours post induction (Fig 5.9a)

Figure 5.9- a). Plot showing specific product concentration in fed-batch fermentation (induction at OD\(_{600}\) of 60). b). 15 % SDS PAGE of total cell lysate showing hGM-CSF expression at various time points in fed-batch fermentation (induction at OD\(_{600}\) of 60).

SDS PAGE analysis of whole cell lysates was done to check hGM-CSF expression levels, which were fairly consistent with the ELISA data and clearly demonstrated that the specific activity \( Y_{P/X} \) of hGM-CSF peaks around 5-6 hours (Fig 5.9b). The product formation rate \( q_p \) was calculated for representative time points by using the equation

\[
q_p = \frac{1}{X} \left( \frac{dP}{dt} \right)
\]

where \( P \) represents volumetric product concentration and \( X \) represents the biomass concentration at that time point.

The initial product formation rate \( q_p \) at the time of induction was 34 mg/g DCW/h which declined to zero within 6 hours of induction. The stress associated with hGM-CSF
expression was the possible reason behind this growth retardation and fall in specific product formation rate.

Interestingly there was only a slight decline in the $q_p$ and maximum $Y_{P/X}$ values when induction was done at an OD$_{600}$ of 60 as compared to the batch run. So it was decided to induce the culture at an even higher OD$_{600}$ and thus obtain better volumetric product concentration. This would also allow us to observe the effects of a lower pre-induction specific growth rate and higher biomass concentration at the time of induction on the expression capacity of the individual cell.

5.3.2 Fed batch cultivation and induction at a cell density of 105 (OD$_{600}$)

Fed-batch cultivation was carried out as described in the previous experiment. The cells were grown in batch mode till an OD$_{600}$ of 13.5 after which a concentrated feed was attached at a feed rate of 15.4 ml/h. The feed was increased exponentially to maintain a specific growth rate of 0.3 h$^{-1}$. Induction was done at a cell OD$_{600}$ of 105 because it was observed that the specific growth rate started declining slowly at higher cell densities and the residual glucose concentration had started rising. Thus the feed rate was kept constant for two hours before induction since excess feeding of substrate can lead to buildup of glucose which in turn can trigger acetate formation. The feeding strategies post induction was to maintain the glucose concentration around 0.1% (100 mg/dl).

From the growth profile it was observed that the pre-induction $\mu$ declined from an initial value of 0.3 h$^{-1}$ to 0.2 h$^{-1}$ at the time of induction. There was a very slight increase in biomass post induction for 30 minutes after which a continuous decline in cell biomass concentration was observed. Thus a final cell OD$_{600}$ of 92 was obtained after 6 hours of cultivation (Fig 5.10a). The specific growth rate ($\mu$) fell from 0.2 h$^{-1}$ to 0.075 h$^{-1}$ within one hour of induction clearly demonstrating the metabolic burden associated with toxic protein expression. It was assumed that all the feed was consumed for maintenance given that there was no increase in biomass and no change in residual glucose concentration. We could thus approximately determine the value of the maintenance coefficient (m) to be $\sim$ 0.1 h$^{-1}$ post induction, which was similar to the data obtained in CSTR studies. There was also a concomitant decline in the substrate uptake rate and thus the feed had to
be reduced to maintain a constant residual glucose level. Similarly the oxygen uptake rate (OUR) fell drastically as could be measured indirectly by looking at the level of gas mixing required. The gas mixing percentage (%) fell from 40 % of pure oxygen to 0 % in six hours demonstrating a drop of more than 3.5 folds in the OUR. Clearly, as observed in the batch run, this was due to loss in cellular viability.

Figure 5.10- a). Time profile of growth (OD₆₀₀), feed rate, pH and residual glucose concentration in fed-batch cultivation of E. coli cells (induction at OD₆₀₀ of 105).

b). Plot showing growth (lnOD₆₀₀) and volumetric product concentration of hGM-CSF in E. coli fed-batch culture (induction at OD₆₀₀ of 105).
From the product profile a continuous increase in volumetric product concentration of hGM-CSF was observed till 5 hours after which it became constant at a level of 3.95 g/l (Fig 5.10b). A maximum specific product concentration of 107 mg/l was achieved which did not change further (Fig 5.11a).

**Figure 5.11**  a). Plot showing specific product concentration in fed-batch fermentation (induction at OD\(_{600}\) of 105). b). 15 % SDS PAGE of total cell lysate showing hGM-CSF expression at various time points in fed-batch fermentation (induction at OD\(_{600}\) of 105).

The above results were also confirmed by gel densitometry done one SDS PAGE (Fig 5.11b). It is apparent from the gel picture (Fig 5.11b) that the recombinant protein buildup increased continuously till 5 hours post induction after which there was no further increase. These results were consistent with the results obtained with ELISA.

The initial \(q_p\) value was 30 mg/g DCW/h which continuously decreased over time to zero in 5 hours post induction. This drop in \(q_p\) was similar to the previous results, however in this case the \(q_p\) values were consistently lower. Similarly the maximum \(Y_{P/X}\) values were lower by \(\sim 10\%\) compared to the product yield in batch and thus the volumetric product concentration was 7 folds higher compared to batch culture. From this result it is clear that the feeding strategy of maintaining a constant residual glucose concentration was able to sustain expression levels even at high cell densities.
The carbon source used for growth can have a considerable influence on cell metabolism and its regulation (Fang and Demain, 1997; Lee et al., 2004). For example acetate accumulation in high cell density cultivation is a common problem when glucose is used. Acetate is produced when the carbon flux in the central metabolic pathway (TCA cycle) exceeds the biosynthetic demand and the capacity for energy generation within the cell (Han et al., 1992). This accumulation can be reduced by controlled addition of carbon source e.g. by glucose limited fed-batch strategies (as in the previous fed-batch runs) or altering the formulation of the growth medium. There are reports demonstrating enhanced expression levels when glycerol was used as a carbon source (Yee and Blanch, 1992). Hence it was decided to conduct a fed-batch experiment using glycerol as the carbon source which had also given slightly better specific product yield in shake flask experiments.

Fed-batch cultivation was carried out like the previous fed-batch runs by substituting glucose with glycerol in the medium. Cells were grown in batch mode in TB glycerol till an OD$_{600}$ of 15. A concentrated feed containing glycerol yeast extract and tryptone was attached at a rate of 15 ml/l which was increased exponentially to maintain a specific growth rate of 0.3 h$^{-1}$. Cells were induced at an OD$_{600}$ of 60 using 0.5 mM IPTG and the growth and product profile was monitored. Samples were taken at regular time intervals of 1 hour. The post induction feed was maintained at constant rate of 80 ml/h till 3 hours after induction since unlike the glucose feed, the biomass concentration increased post induction for 3 hours. The average post induction $\mu$ for these 3 hours was 0.07 h$^{-1}$. The feed was decreased in steps only when the cells stopped growing i.e. after 4 hours of induction (Fig 5.12b). A maximum cell OD$_{600}$ of 80 was obtained after 4 hours which declined marginally with induction time to 78 (Fig 5.12a).
A continuous increase in volumetric product concentration was obtained which became constant at 3.5 g/l after 4 hours of induction (Fig 5.12b). A similar pattern was obtained with the specific product concentration ($Y_{P/X}$) where it reached a maximum level of 112
mg/g DCW after 5 hours of induction which is quite close to the value obtained in TB glucose medium (118 mg/g DCW) for 60 OD<sub>600</sub> induction (Fig 5.13a)

![Graph showing specific product concentration in fed-batch fermentation with TB glycerol medium (induction at OD<sub>600</sub> of 60).](image)

![Image showing 15% SDS PAGE of total cell lysate showing hGM-CSF expression at various time points in fed-batch fermentation in TB glycerol medium (induction at OD<sub>600</sub> of 60).](image)

**Figure 5.13- a)**. Plot showing specific product concentration in fed-batch fermentation with TB glycerol medium (induction at OD<sub>600</sub> of 60). **b)**. 15% SDS PAGE of total cell lysate showing hGM-CSF expression at various time points in fed-batch fermentation in TB glycerol medium (induction at OD<sub>600</sub> of 60).

The whole cell lysate was analyzed on 15% SDS PAGE (Fig 5.13b). The expression levels rose quickly to reach a maximum value within 3-4 hours of induction confirming the results obtained with ELISA.

The specific product formation rate (q<sub>p</sub>) was calculated and a high initial q<sub>p</sub> value of 47 mg/g DCW/h was obtained which gradually decreased to zero in 5 hours. This high initial value of q<sub>p</sub> could be because of glycerol in the culture medium which is considered to be less catabolite repressive than glucose (Sutton and Freundlich 1980).

### 5.3.4 Discussion

The major success of the fed-batch experiments can be gauged from the q<sub>p</sub> vs time profile post induction. It is clear that there was very little change in the pattern as well as the absolute q<sub>p</sub> values for the different fed-batch experiments in comparison with batch culture (Fig 5.14). This ensured very little change in the time averaged q<sub>p</sub> values post
induction which resulted in similar levels of protein buildup inside the cell. Thus the volumetric product concentration increased concomitantly with the increase in biomass concentration with the maximum value being 3.95 g/l when induction was done at 105 OD$_{600}$. These results compare favourably with previous reports of hGM-CSF expression where typical product concentrations are in the milligram range. The maximum $q_p$ was obtained with glycerol medium where the initial values were 47 mg/g DCW/h. However the major problem with glycerol is the lack of on-line monitoring facility which does not allow a feed back control of the feeding rate. Thus a non-optimum feeding profile post induction could be the reason behind the quicker drop in $q_p$ compared to glucose fed cultures.

Interestingly even though the initial $q_p$ values were lower for the glucose fed-batch cultures (as compared to batch culture), these could be sustained for the longer time periods. Clearly feeding of glucose and complex nitrogen sources helped to sustain cellular viability and hence expression rates. Given that negligible growth was obtained post induction for the fed-batch cultures, we can see that there is a physiological state when recombinant protein expression continues while growth stops. Though this is a transient phenomenon, it is worth exploring since in this situation all the metabolic fluxes are directed towards foreign protein expression.

![Figure 5.14](image_url)