CHAPTER 7.

BIOPROCESS STRATEGIES FOR hGM-CSF PRODUCTION IN Pichia Pastoris
7. Bioprocess strategies for hGM-CSF production in *Pichia pastoris*

After optimizing expression levels in shake flasks it was decided to go for bioreactor studies where the kinetics of growth and product formation could be studied and high cell density cultivation strategies could be optimized.

7.1 Batch fermentation

Batch cultivation was done in a bioreactor where the pH was controlled at 5 and dissolved oxygen at 40%. Cells were grown in the previously optimized YPDCA medium at 30°C. The cells grew exponentially with a maximum specific growth rate ($\mu$) of 0.2 h$^{-1}$ up to 18 hours, which decreased in the later phase of cultivation. A final cell biomass of 21 g DCW/L (OD$_{600}$=57.5) was obtained after 26 hours of cultivation. From the product profile a continuous increase was observed in the extracellular concentration of hGM-CSF. A maximum hGM-CSF concentration of 54 mg/L of hGM-CSF was obtained after 22 hours of cultivation which was similar to the shake flask results. The controlled conditions of growth allowed us to reduce the cultivation time to less than half in comparison to shake flask culture.

From the pattern of growth and hGM-CSF production, it appeared that product formation was growth associated (Fig 7.1). It was observed that the expression rates were maximum during the log phase of cultivation. Therefore another batch fermentation with a higher concentration (2X YPDCA) of batch medium was done to observe the effects of extended log phase on cell growth and product formation. A maximum product concentration of 95 mg/L and a final biomass of 38 g DCW/L (OD$_{600}$=104) was obtained after 26 hours of cultivation (Fig 7.1b). Thus both biomass and product concentration increased 1.76 fold leading to a similar specific product yield of ∼2.5 mg/g DCW in both batch runs (Fig 7.1). However the overall biomass yield ($Y_{X/S}$) was lower by ∼10% when cultivation was done in 2X medium.
7.2 Continuous cultivation studies

Continuous culture techniques are an invaluable tool to estimate the stoichiometric and kinetic parameters of growth and product formation. Typically steady state values of biomass and product concentration at different dilution rates can give us a fairly precise estimate of these parameters. However, recombinant cultures tend to demonstrate high levels of genetic instability especially under induced conditions. This prevents the establishment of a true steady state and transient state continuous culture studies need to be conducted as was done with *E. coli* in chapter 5. Fortunately, the GAP promoter based expression system is not only constitutive but also extremely stable (given that the gene is chromosomally integrated) and this allowed the establishment of steady state which could then be used to determine the growth and product formation kinetics.

One of the problems with the use of complex media is the inability to accurately determine the residual substrate concentration in the culture medium. This makes it extremely difficult to determine the stoichiometry of product and biomass formation \((Y_{PX}, Y_{XS})\). While this problem is easily rectified by using minimal or defined media,

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**Figure 7.1-** Profile of biomass and hGM-CSF production in batch cultivation of recombinant *Pichia pastoris*.  
**a).** 1X YPDCA medium  
**b).** 2X YPDCA medium.
this option was not available to us since we wanted to study the kinetics of this system when it was producing large amounts of hGM-CSF. This was only possible in complex media as had been shown in previous shake flask studies. To circumvent this problem and also to study the behavior in high cell density culture (which would be more relevant for designing of fed-batch strategies), it was decided to conduct continuous cultivation not only at different dilution rates but also at different media concentrations (1X, 2X and 4X). Given the basic rationale of continuous culture at steady state, the residual substrate concentration is solely determined by the dilution rate \( D = \mu = \mu_{\text{max}}S/(K_s+S) \Rightarrow S = DK_s/(\mu_{\text{max}}-D) \). Therefore a higher inlet substrate concentration serves only to increase the steady state biomass concentration given by \( X = Y(S_0 - S) \) where \( S_0 \) is the inlet substrate concentration and \( S \) is the steady state concentration. The ratio of the levels of biomass obtained with different inlet substrate concentrations can therefore be used to both indirectly estimate the residual substrate concentration and also to see the effect of cell density on the production capacity of the individual cell (i.e. specific productivity; \( q_p \)).

7.2.1 CSTR operation

The \( P. \) \textit{pastoris} culture was grown in batch mode in 1X, 2X and 4X YPDCA medium (1X YPDCA for CSTR contained; 1% glucose, 1% peptone, 0.5% yeast extract and 0.5% casamino acids) respectively for the three different runs as described earlier. Interestingly, though the cells showed an extended log phase, when inoculated in rich media, they grew reasonably well in all 3 media concentrations. The pH was maintained at 5 and DO at 40%. The cells were allowed to grow to mid log phase before switching on the inlet and outlet pumps to get the desired dilution rates of 0.2 \text{h}^{-1}. This was done to ensure that the cells were in the best physiological condition to quickly adapt to the continuous culture growing at the desired \( \mu \) value. Thus the inlet pump was switched on at a flow rate of 200 ml/h (reactor operating volume = 1L) when the \( \text{OD}_{600} \) was 18, 42, and 75 for 1X and 2X and 4X medium respectively. The outlet pump was attached to an overflow device to ensure that the volume remained constant. Three reactor volumes were passed to confirm the establishment of steady state. The \( \text{OD}_{600} \) stabilized at 25, 49.
and 93 for 1X, 2X and 4X medium respectively. Simultaneously other parameters like dissolved oxygen concentration (DO) and residual glucose concentration was measured to confirm the steady state.

Three samples were taken in duplicate over a 5-8 hours period to check the reproducibility of the data and demonstrate that these are invariant over time. The dilution rate was then reduced in steps of 0.025 h\(^{-1}\) (i.e. from 0.2 h\(^{-1}\) to 0.175 h\(^{-1}\) and then subsequently to 0.15 h\(^{-1}\) and so on). After each reduction two reactor volumes of feed were passed to ensure steady state. It was observed that since the new steady state was quite close to the previous one, it got established fairly quickly (within one reactor volume) and thus we did not need to pass three reactor volumes of feed for every new steady state. Samples were collected over a 5-8 hours period to demonstrate time invariance. Finally this sequence of slowly lowering the dilution rate (from 0.2 h\(^{-1}\) to 0.25 h\(^{-1}\)) was reversed and dilution rate was increased step wise back to 0.2 h\(^{-1}\). The same steady state values of biomass and product were obtained demonstrating that there was no significant emergence of non-expressing cells in the culture even after the passage of a large number of generations.

### 7.2.2 Growth kinetics:

An indirect method was used for the estimation of residual substrate concentration from the plot of biomass concentration at steady state (Fig 7.2a). Firstly the residual glucose concentration was estimated by a hand held glucometer and it was observed that they were typically in the range of 50-80 mg/dl. Since the inlet glucose concentration was 10-40 g/l (i.e. 1000-4000 mg/dl). It was clear that most of the glucose had been consumed for growth. To estimate the residual presence of other carbon source (since the feed contained complex nitrogen sources) the \(Y_{XS}\) value were calculated on the basis of glucose consumption alone i.e. \(Y_{XS} = \bar{X}/(S_0 - \bar{S}) \approx \bar{X}/S_0\) (since most of the glucose had been consumed). The values of \(Y_{XS}\) obtained using the above equation were almost identical (\(\sim 0.9\) g DCW/g glucose) for different inlet feed concentrations (for a given dilution rate) (Fig 7.2b). This implied that the biomass concentration increased proportionally to inlet substrate concentration thereby demonstrating that all the substrate had been consumed.
for growth. It was therefore clear that substrate utilization was extremely efficient at all dilution rates and thus if we use a Monod model for growth kinetics, the $K_s$ values would be very small. The values of $Y_{XS}$ ranged between 0.7-0.9 g DCW/g glucose which is similar to the values reported in literature when other complex nitrogen sources are available in the media (Mendoza-Vega et al., 1994). The $Y_{XS}$ values were slightly lower when 4 X media was used which could possibly be because of buildup of by-products in the high cell density cultures. Since this biomass yield ($Y_{XS}$) declined at lower dilution rates, a double reciprocal plot between $1/Y_{XS}$ vs $1/D$ was plotted to estimate the maintenance coefficient. Interestingly we did not obtain a straight line which shows that the maintenance coefficient was not a constant (Fig 7.3). At low dilution rates, there was very little change in the yield coefficient ($Y_{XS}$) possibly because (as shown later), the cells do not produce a significant amount of product. However at higher dilution rates (where cells produce substantial amounts of product) there was a significant change in biomass yield coefficient giving us a ‘m’ value of 0.05h$^{-1}$ (Fig 7.3). The pattern of change in biomass yield coefficient ($Y_{XS}$) was identical in all three cases (Fig 7.2b). This allowed us to estimate the value of the maintenance coefficient which was invariant over the three different runs.

![Figure 7.2- Profile showing a). dry cell weight and b). specific biomass yield in continuous cultures of Pichia pastoris at different dilution rates and media concentrations.](image-url)
7.2.3 Product formation kinetics:

The extracellular product concentration at steady state was measured at different dilution rates for different inlet substrate concentrations (Fig 7.4a). Given the product balance equation at steady state is $q_p\bar{x} = D\bar{p}$ we can define the $\bar{p}/\bar{x}$ values at steady state as the product yields coefficient ($Y_{P/X}$). It is well known that $Y_{P/X}$ values remain constant over different dilution rates when the product is completely growth associated while it declines with increasing dilution rates ($D$) when it is partly growth associated and partly non-growth associated (Luedeking and Piret 1959). These $Y_{P/X}$ values were plotted against different dilution rates and interestingly we observed a slight increase in $Y_{P/X}$ values when $D$ was increased. This showed that the increase in $q_p$ was faster than $\mu$ and it can be claimed that product formation was slightly more than growth associated (since $\mu = D$ at steady state). This, more than growth associated nature, of product formation kinetics is typical for many different recombinant proteins (Curless et al., 1990; Babu et al., 2000; Srivastava et al., 2005). High cell density culture also did not have a significant effect on
the production capacity of the individual cell. Thus $Y_{P/X}$ values in high cell density culture were 80-90% of the $Y_{P/X}$ values at low cell density (Fig 7.4b). This clearly shows that by-product formation did not have a major effect on product formation.

![Figure 7.4- Profile showing a). Volumetric product concentration and b). specific product yield ($Y_{P/X}$) of hGM-CSF in continuous cultures of Pichia pastoris at different dilution rates and media concentrations.]

The slight increase in the product concentration at higher dilution rates was confirmed by running steady state samples of the product i.e., extracellular hGM-CSF at different dilution rates on SDS PAGE (Fig 7.5)

![Figure 7.5- 15% SDS PAGE showing the extracellular expression of hGM-CSF at different dilution rates in 4X medium. G: Glycosylated hGM-CSF, U: Unglycosylated hGM-CSF]

To emphasize the more than growth associated nature of product formation, the specific productivity ($q_p = D \cdot Y_{P/X}$) was plotted as a function of different dilution rates for all the
three media concentrations (Fig 7.6). The slightly concave nature of this plot is an indicator that \( q_p \) increased marginally faster than \( \mu \) (or D). We thus observe that the constitutive GAP promoter was primarily growth associated and changes in the physiological culture conditions of the cells brought about by changing the cell density did not affect this system. This provided us with a very obvious rationale for the optimization of product formation whereby the attainment of the maximum biomass in the shortest time period (maximum specific growth rate) would automatically maximize the productivity of the culture.

*Figure 7.6-* Plot showing the product formation rate of hGM-CSF at different dilution rates and media concentrations.
7.3 Fed-batch cultivation

Since product formation appeared to be growth associated a higher biomass would help in achieving higher product concentrations. Fed-batch studies were carried out with the above objective, where three different feed compositions viz. defined feed (containing glucose and salts), semi defined feed (additionally containing casamino acids) and complex feed (15X YPDCA) were compared. The composition of batch and feed medium has been given in material and method section (Table 3.3)

7.3.1 Defined feed medium

Batch cultivation was done in YPDCA and feed was initiated at a cell density of 18 g DCW/L (OD\textsubscript{600} = 50) to maintain a specific growth rate of 0.2 h\textsuperscript{-1}. However we observed a gradual decline in the specific growth rate and after 26 hours of cultivation a maximum cell density of 45 g DCW/l (OD\textsubscript{600} = 123) with a biomass yield coefficient (Y\textsubscript{X/S}) of 0.62 g DCW/g glucose was obtained in the fed batch phase (Fig 7.7). The value of Y\textsubscript{X/S} was lower than what was obtained in continuous culture because the feed medium contained only glucose and salts and thus the ratio of complex nitrogen sources to glucose in the present case was effectively lower. The starvation of these nitrogen sources is a possible reason for the slow decline in specific growth rate (\(\mu\)).

**Figure 7.7**- Profile of biomass (OD\textsubscript{600}) and extracellular hGM-CSF production during fed-batch cultivation of recombinant *Pichia pastoris* using defined feed containing glucose and salts. The lnOD\textsubscript{600} vs time profile was used to estimate the specific growth rate.
The maximum hGM-CSF concentration was 104 mg/l and thus the specific product yield was 2.3 mg/g DCW, slightly lower than what was obtained in batch culture. The extended late log phase where the $\mu$ declined from 0.2 h$^{-1}$ to zero could be the reason behind this slight decline in $Y_{P/X}$ similar to the CSTR results where $Y_{P/X}$ value fell slightly at lower dilution rates.

### 7.3.2 Semi defined feed medium

In this case, batch cultivation was done in YPDCA medium and a semi defined feed was attached at a biomass concentration of 17 g DCW/l ($OD_{600} = 47$) after 15 hours of cultivation to get a specific growth rate of 0.2 h$^{-1}$. Using this feed, we were able to prevent a decline in the specific growth rate for 22 hours, which helped in achieving a maximum biomass of 60 g DCW/l ($OD_{600} = 165$) in 27 hours of cultivation. A higher final product concentration of 145 mg/l was obtained (Fig 7.8). A fairly sharp decline in $\mu$ was observed after 22 h demonstrating the starvation of some critical nutrients. No further increase in biomass or product concentration was obtained after this period. Thus we observed a 1.4 fold increase in the product concentration with a specific product yield ($Y_{P/X}$) of 2.4 mg/g DCW.

![Figure 7.8- Profile of biomass (OD$_{600}$) and extracellular hGM-CSF production during fed-batch cultivation of recombinant *Pichia pastoris* using semi defined feed supplemented with casamino acids. The lnOD$_{600}$ vs time profile was used to estimate the specific growth rate.](image)
7.3.3 Complex feed medium

Complex feed was used in order to get an extended log phase and hence increase the final biomass concentration, so as to get the maximum concentration of the extracellular product. Batch cultivation was done in YPDCA medium and feed was attached when the biomass concentration reached 16 g DCW/l (OD<sub>600</sub> = 44). We observed that the specific growth rate of 0.2 h^{-1} was maintained for a much longer period and thus the maximum biomass and hGM-CSF concentrations obtained were 98 g DCW/l (OD<sub>600</sub> = 265) and 250 mg/l respectively (Fig 7.9). The biomass yield coefficient (Y<sub>X/S</sub>) was 0.8 g DCW/g glucose primarily because of the additional availability of complex nitrogen sources. Thus the hGM-CSF production was 2.5 and 1.75 folds higher as compared to glucose feed and semi defined feed respectively. Interestingly the specific product yield (Y<sub>P/X</sub>) remained unchanged at 2.4 mg/g DCW clearly demonstrating that higher product levels are achieved primarily through enhancing biomass concentrations and there is no effect on the expression ability of the individual cell.

![Figure 7.9](image_url)  
**Figure 7.9**- Profile of biomass (OD<sub>600</sub>) and extracellular hGM-CSF production during fed-batch cultivation of recombinant *Pichia pastoris* using complex feed (15X YPDCA). The lnOD<sub>600</sub> vs time profile was used to estimate the specific growth rate.
The SDS-PAGE (Fig 7.10a) and immunoblot (Fig 7.10b) revealed that there were very few contaminating proteins present in the supernatant, which in turn would facilitate purification by simple chromatographic techniques. Western blot showed a differentially glycosylated hGM-CSF in the range 27-45 kDa and also a faint non-glycosylated form at 15 kDa which was similar to previous reports (Wu et al. 2003; Miyajima et al. 1986).

**Figure 7.10** - 15 % SDS-PAGE and Western blot of the culture supernatant of fed-batch culture with complex feed demonstrating hGM-CSF expression.

a) Lane 1: Protein molecular weight marker (Mid-range). Lanes 2-9: 15 μl of culture supernatant at various time points of cultivation.

b) Western blot of the culture supernatant. Lanes 1-8: 15 μl of culture supernatant at various time points of cultivation

c) 15 % SDS PAGE analysis of deglycosylation of hGM-CSF.

Lane 1: Protein Molecular weight marker, Lane 2: 15 μl culture supernatant after 34 h of cultivation, Lanes 3-6: hGM-CSF from culture supernatant of 28, 30, 32 and 34 h of cultivation after deglycosylation. G: Glycosylated form of hGM-CSF, D: Deglycosylated form

The protein sample was deglycosylated with N-glycosidase (PNGase F) and run on SDS-PAGE (Fig 7.10c). A major protein band of ~15 kDa corresponding to the unglycosylated
form of hGM-CSF was observed conclusively demonstrating that very few contaminating proteins were present in the culture supernatant.

We were thus able to successfully increase the extracellular hGM-CSF concentration 5 folds in comparison to batch culture and also achieved a very high volumetric productivity of 7.35 mg/l h⁻¹ given the short fermentation times.

7.3.4 Discussion

From the growth and product formation profiles obtained during the batch, continuous and fed-batch culture it is clear that product formation was strongly growth associated. As a matter of fact the \( Y_{p/x} \) values actually increased slightly at higher \( \mu \) values demonstrating the ‘more than growth associated nature’ of product formation. This kind of product formation kinetics is a typical feature of the constitutive GAP promoter which allows the design of robust feeding strategies, unlike the inducible AOX1 promoter where the concentration of methanol in the media has a critical role in determining the level of expression (Murray and Duff 1989). However it is important to note that growth associated product formation kinetics does not guarantee constancy in the values of \( Y_{p/x} \) while expressing different recombinant proteins. Thus recombinant exo-levanase had a \( Y_{p/x} \) value of 7.7 mg/g DCW (Menandez et al. 2004) while rec-chitinase had a \( Y_{p/x} \) of 3 mg/g DCW when it was grown in high cell density continuous culture at a very low specific growth rate (Goodrick et al. 2001). The lower \( Y_{p/x} \) values obtained with hGM-CSF possibly reflects the “difficult to express” nature of this protein which is why the earlier reported yields of hGM-CSF is uniformly poor across various systems. The major advantage of this expression system was its ability to efficiently secrete the protein to the extracellular medium which provides many advantages. Firstly the potential toxic effects of hGM-CSF were absent and cell growth was not hampered. Secondly very few contaminating proteins were present in the extracellular medium which would aid downstream processing and also the problem of proteolytic degradation was absent allowing the build up of the protein concentration in the extracellular medium. This bioprocess strategy can easily be extended to the expression of other difficult to express recombinant proteins.