CHAPTER 5.

RESULTS & DISCUSSION

(BIOREACTOR STUDIES)
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Batch cultivation

Batch cultivation using PNHAsp cells (pPNHAsp vector in BLR (DE3) cells) was done to test the level of recombinant asparaginase expression in a bioreactor where cultivation parameters like pH, D.O (dissolved oxygen) etc. are better controlled than in shake flasks. A standard 2-litre bench top reactor (Infors AG, CH4103) was set up with a 1 litre working volume. The batch media used was TB (with 0.4% glycerol). The media was aseptically inoculated with 100 ml of overnight grown culture of PNHAsp cells in TB medium. Ampicillin was used at a concentration of 200 μg/ml (2X). The temperature, pH and the dissolved oxygen (D.O) was set at 37°C, 7.2 and 40% respectively with an initial minimum stirrer speed of 200 rpm and RPM based control of D.O at the set point.

The airflow was set at 1 vvm and not changed during the fermentor run. The pH was controlled automatically with the dosing pumps using 1N NaOH and 1N HCl.

Fig 5.1 - Time profiles of growth (OD_{600}, LnOD_{600}) and asparaginase activity in the culture supernatant and the periplasmic fractions. Arrow indicated the point of induction using 0.1 mM IPTG.
D.O control was set at 40% until the agitation rate reached 1000 rpm after which if required automatic pulsing of pure O₂ was done to maintain the D.O. Excess foaming was controlled by addition of antifoam. Cells grew to an OD₆₀₀ of 7.3 in 4.5 hours at which point the culture was induced with 0.1 mM IPTG. Samples were collected at defined time intervals till 24 hours post-induction and analysed for asparaginase expression (activity assay and SDS-PAGE gel) in both extracellular and periplasmic fractions.

Cells grew with a pre-induction specific growth rate of 0.7 h⁻¹ which fell drastically within 2 hours of IPTG induction, and the biomass concentration did not increase any further after 2 hours (Fig 5.1). A final biomass concentration of ~ 4 g/L (DCW) corresponding to an OD₆₀₀ of 10.4 was obtained 24 hours post-induction. Periplasmic expression levels increased till 12 hour post-induction reaching a maximum of 12.5 Units/ml and then declined to a final yield of 7 Units/ml. However there was a constant build-up in the levels of recombinant asparaginase in the culture media throughout the cultivation period (Fig 5.2) resulting in a maximum yield of ~ 22.25 Units/ml at the end of cultivation.

This growth pattern and activity profile was consistent with the shake flask results where a similar asparaginase yield (~ 20.5 Units/ml) was obtained in the extracellular fraction. However the specific product yield (Activity/OD₆₀₀/ml) in the batch cultivation was much lower at 2.2 in comparison to 3.72 achieved in shake-flasks.

Even though no growth was observed 2 hour post-induction, the cells appeared to be in a metabolically active phase which was evident from the gradual fall of RPM even after stoppage of growth {RPM was a useful on-line indicator of the
oxygen transfer rate (OTR), which matches the oxygen uptake rate (OUR) when D.O is at a steady state. The decline in OUR over the period of post-induction cultivation (as indicated by drop in RPM) was possibly due to substrate starvation which in turn could be the reason behind the lower specific product yields. This clearly shows that availability of nutrients was a critical factor in the production capability of cells even though growth was not observed during the production phase. Therefore to maintain a continuous supply of nutrients as well as achieve higher cell densities fed-batch studies were under taken.

**Fed-batch cultivation**

Since recombinant protein production in *E. coli* can be significantly increased through the use of high cell density cultivation techniques, we decided to optimize the bioprocess strategy for the scale up of our expression system in a bioreactor employing fed-batch techniques for high cell density cultivation (Lee SY 1996; Riesenberg and Guthke 1999). The effect of the time of induction, specific growth rate and D.O on the expression levels of recombinant proteins has been studied in *E. coli* fed-batch fermentation processes (Jeong and Lee 1999; Kwon et al. 1996; Hellmuth et al. 1994). It has been shown that the biomass concentration at the time of induction can have a considerable effect on the final expression yields achieved (Jeong and Lee 1999; Kwon et al. 1996).

In the case of extracellular asparaginase expression we had observed that within a few hours post-induction the specific growth rate (μ) had reached close to zero. However cellular health remains an important factor in the product forming ability and this also depends upon the pre-induction growth rate. The standard strategy followed by many researchers to enhance recombinant protein production is to maintain a high specific growth rate especially pre-induction while running high cell density cultures (Ryan et al. 1996). This helps in maintaining optimum cellular health which is required to overcome the metabolic stress associated with recombinant protein expression (Sanden et al. 2003). Post-induction growth
typically is an intrinsic property of the expression host system which gets reduced in a programmed manner upon induction and often cannot be controlled by external feeding (Panda et al. 1999). It has also been reported E. coli possesses a more efficient protein synthesis machinery (and thus a greater potential for expressing foreign proteins) when growing at higher growth rates (Curless et al. 1990). A specific growth rate of 0.3 h\(^{-1}\) is considered high enough to maintain good cellular health and at the same time is low enough not to allow acetate accumulation especially when using complex medium. Shokri et al. (2002) have also shown that the specific growth rate has a direct effect on export of the periplasmic proteins into the medium, with the optimum export taking place at a specific growth rate of 0.3 h\(^{-1}\). The structural properties of the inner and outer membranes of E. coli are critical to the export efficiency and it is argued that at a specific growth rate of 0.3 h\(^{-1}\) the highest membrane fluidity is obtained leading to a higher efficiency of protein export to the medium. Additionally a relatively higher specific growth rate of around 0.3 h\(^{-1}\) prevents several negative effects such as: increased lysis, higher levels of endotoxin accumulation, membrane stiffness, which are characteristic of cells at low specific growth rates (Arneborg et al. 1993; Shokri et al. 2002; 2003). Furthermore, the fed-batch and continuous cultivation studies on characterization of membrane structure and function in E. coli it seems evident that there exists a growth dependent switch point in the membrane regulation at a specific growth rate of approximately 0.3h\(^{-1}\) (Shokri and Larsson 2004). An exponential feeding strategy to maintain a specific growth rate of 0.3 h\(^{-1}\) was therefore used for the pre-induction period. Also cultivation at lower growth rate often results in change in shape of the cells, which were shown to become smaller and more round which also influenced the production ability of the cells (Prytz et al. 2003).

One common concern in conducting high cell density cultivation towards higher specific growth rates is the formation of acetate, which on accumulation during the run (to inhibitory levels) becomes toxic to cells and limits the level of cell density achieved as well as the level of gene expression (Doelle et al. 1982;
Jensen and Carlsen 1990; Han et al. 1992; Kleman and Strohl 1994). Previous shake-flask studies on comparison between glucose and glycerol (as carbon source) on acetate formation have revealed that use of glycerol resulted in only one-third of the level of acetate produced in comparison to glucose. Presumably formation of lower levels of acetate on glycerol is one of the reasons for lower inhibitory effects of glycerol even at higher concentrations during a fed-batch culture (Ko et al. 1994). High concentration of glycerol in media has been shown to increase the soluble protein yield by five-fold during over-production of recombinant β-lactamase (Hong 1992). Glycerol is an osmolytic stabilizer probably due to its ability to enter into and strengthen the water lattice structure and at high concentrations glycerol is also known to lower the rates of aggregation producing solute interactions (Schein 1990). Moreover, shake-flask studies for extracellular asparaginase expression have also shown that higher recombinant product yields in the media were obtained using glycerol than glucose as carbon source. Therefore, the following fed-batch studies were done with glycerol as the primary carbon source.

Earlier studies done by us (data not shown) have demonstrated that a specific growth rate of 0.3 h⁻¹ can be sustained using a combination of TB media and exponential feeding strategy to an \( \text{OD}_{600} \) of ~150 if induction is not done. However after this the culture specific growth rate starts declining possibly due to the accumulation of by products in the medium.

Since shake-flask results showed that delayed induction gave better yields it was decided to examine the effect of the time of induction on extracellular production of recombinant asparaginase, cells were grown at a specific growth rate 0.3 h⁻¹ by exponential feeding strategy and induced with 1 mM IPTG at varying \( \text{OD}_{600} \) values of 33, 60, 90 and 135.
Fed-batch with induction at low cell density (OD₆₀₀ of 33)

Fed-batch cultivation was carried out in a 2L computer controlled Infors AG fermentor. Batch media was buffered TB media supplemented with 200 μg/ml ampicillin, pH was controlled at 7.2 by addition of 2N HCl/NaOH. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate of 0.3 h⁻¹.

Expression of recombinant asparaginase was induced by addition of 1mM IPTG (final concentration) that was maintained in the culture medium during the course of cultivation by incorporating IPTG in the feed. The feed was increased exponentially till the period of increasing biomass post induction. Automatic D.O control was set and maintained at 40% throughout the cultivation.
It was observed that cells stopped growing within 6 hours of induction (fig 5.3). However product formation continued even though the cells had stopped growing and even after 24 hours post induction the product concentration was found to be increasing in the culture supernatant though at a slightly slower rate (Fig 5.4). A maximum cell OD$_{600}$ of 48 corresponding to a dry cell weight (DCW) of 18.28 g/L was obtained 24 hours post induction. A final volumetric yield of $3.9 \times 10^5$ Units/L of recombinant asparaginase was obtained with a specific activity of $2.1 \times 10^4$ Units/g DCW/L. The extracellular recombinant protein constituted 75% of the total extracellular protein at 12 hours that then decreased to 69% after 24 hours of induction.

**Fed-batch with induction at intermediate cell density (OD$_{600}$ of 60)**

Fed-batch cultivation was carried out as described earlier. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate of 0.3 h$^{-1}$. Expression of recombinant asparaginase was induced by addition of 1mM IPTG (final concentration) at an OD$_{600}$ of 60.0. The feed was increased exponentially till the period of increasing biomass post induction. Automatic D.O control was set and maintained at 40% throughout the cultivation.
It was observed that cells stopped growing within 4 hours of induction (Fig 5.5). Cells induced at an intermediate cell OD<sub>600</sub> of 60.0 gave a final OD<sub>600</sub> of 78, corresponding to a dry cell weight of 30 g/L at 24 hours of post induction. The final volumetric yield was 5.7 X 10<sup>6</sup> Units/L with a specific activity of 1.9 X 10<sup>4</sup> Units/g DCW/L. The extracellular recombinant protein constituted 71.5% of the total extracellular protein at 12 hours post induction that declined to 62% after 24 hours of induction.
Fed-batch with induction at higher cell density (OD\textsubscript{600} of 90)

Fed-batch cultivation was carried out in a 2L computer controlled Infors AG fermentor as described earlier. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate (\(\mu\)) of 0.3 h\(^{-1}\). Expression of recombinant asparaginase was induced by addition of 1mM IPTG (final concentration) at an OD\textsubscript{600} of 90.0. The feed was increased exponentially till the period of increasing biomass post induction. Automatic D.O control was set and maintained at 40% throughout the cultivation.

It was observed that cells stopped growing within 2.5 hours of induction (Fig 5.7). Cells induced at a higher cell OD\textsubscript{600} of 90.0 resulted in achieving a final OD of 119 corresponding to a dry cell weight of 45 g/L, twenty-four hours after
induction. A final volumetric yield of $8.7 \times 10^5$ Units/L was obtained with a specific activity of $1.9 \times 10^4$ Units/g DCW/L. The extracellular recombinant protein constituted 69% of the total extracellular protein at 12 hours post induction that decreased to 58% after 24 hours of induction (Fig 5.8).

Fed-batch with induction at highest cell density ($OD_{600}$ of 135)

Fed-batch cultivation was carried out in a 2L computer controlled Infors AG fermentor as described earlier. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate ($\mu$) of 0.3 h$^{-1}$. Expression of recombinant asparaginase was induced by addition of 1mM IPTG (final concentration) at an $OD_{600}$ of 135.0. The feed was increased exponentially till the period of increasing biomass post induction. Automatic D.O control was set and maintained at 40% throughout the cultivation.

It was observed that cells stopped growing within an hour of induction (Fig 5.9). Cells induced at the highest cell density tested i.e. an $OD_{600}$ of 135.0 resulted in negligible post induction growth and an $OD_{600}$ of 141 corresponding to a final dry cell weight of 53.5 g / L was obtained twenty-four hours after induction.

A Final volumetric yield of $5.8 \times 10^5$ Units/L was obtained which is reflected in a sharp drop in specific activity of $1.1 \times 10^4$ Units/g DCW/L. The extracellular recombinant protein constituted 54% of the total extracellular protein at 12 hours that further decreased to 42% after 24 hours of induction.
We thus observed a progressive decline in the specific activity as the induction OD was increased. However this drop was critical at the highest OD leading to a fall in volumetric activity. We thus observe that pre-induction specific growth rate is not the only parameter which determines the expression ability of the individual cell. Increasing biomass possibly leads to the accumulation of by products which can play a critical role in determining the specific productivity even when it does not hamper specific growth rate. We thus observed the cessation of growth a few hours post induction, this period of increase in biomass post induction became progressively shorter with increasing OD$_{600}$ of induction, indicating that cellular health and

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**Fig 5.9** - Time profiles of growth (OD$_{600}$, DCW), total extracellular protein and the asparaginase production in the culture supernatant during fed-batch cultivation. Arrow indicated the point of induction at an OD$_{600}$ of 135 with 1 mM IPTG.

**Fig 5.10** - 12% SDS-PAGE showing asparaginase production profile in culture supernatant at various hours post-induction during high cell density cultivation. Induction was done at an OD$_{600}$ of 135.
hence the ability to grow with the metabolic stress associated with recombinant protein expression was better at lower OD's. However the most interesting observation was that recombinant protein production continued unabated even after growth stopped. Thus this system is able to divert all the metabolic fluxes towards product formation and not to growth. The specific product formation rate was maximum (893 Units/g DCW/h) where induction was done at the lowest cell density (OD$_{600}$ =33), which declined with induction at higher OD's. Induction at the highest cell density (OD$_{600}$ =135) resulted in a sharp decline in the specific product formation rate (455 Units/g DCW/h), which was the reason for achieving lower specific and volumetric activities even though the biomass level obtained was higher. The maximum volumetric activity was achieved when induction was done at an OD$_{600}$ of 90, where the specific protein production rate was slightly lower (798 Units/g DCW/h) but the product of specific protein production rate and biomass concentration was the highest. Thus a final volumetric activity of 8.7 X 10$^5$ Units/L corresponding to ~ 5.24 g/L of recombinant asparaginase was achieved in the extracellular medium. This constituted about 58% of the total protein present in the culture medium.

It is important to note that the extracellular asparaginase levels were continuously increasing even after 24 hours post-induction, at which point the bioreactor run was terminated. It is thus possible to increase the extracellular yield further by running the bioreactor for a longer period of time especially if one were to use dialysis reactors (Portner and Markl 1998; Fuchs et al. 2002) where the by products and the asparaginase would be continuously removed from the system.

**Effect of D.O on the extracellular expression of recombinant asparaginase**

Fed-batch cultivation was carried at 20 % D.O and induction was done at higher cell density (OD$_{600}$ of 94) to study the effect of lowering of the D.O on extracellular expression. Previous reports on extracellular expression have
shown that D.O concentration variation has had a major effect on the final product yields (Kleist et al. 2003).

Fed-batch cultivation was carried out in a 2L computer controlled Infors AG fermentor. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate of 0.3 h⁻¹. Expression of recombinant asparaginase was induced by addition of 1mM IPTG (final concentration) that was maintained in the culture medium during the course of cultivation. The feed was increased exponentially till the period of increasing biomass post induction.

![Fig 5.11](image-url) - Time profiles of growth (OD₆₀₀, DCW), total extracellular protein and the asparaginase production in the culture supernatant during fed-batch cultivation with D.O maintained at 20% in the post-induction period. Arrow indicated the point of induction at an OD₆₀₀ of 94 with 1 mM IPTG.

Automatic D.O control was set and maintained at 40% in the pre-induction period and which was reduced and maintained at 20% in the post-induction period. Cells were induced at an intermediate cell density of 94 with 1 mM IPTG.

It was observed that cells stopped growing within 2-3 hours of induction. Cells induced at a higher cell density of 94 resulted in achieving a final OD₆₀₀ of 121 corresponding to a final dry cell weight of 45.2 g/L, twenty-four hours after
induction. A final volumetric yield of $6.45 \times 10^5$ Units/L was obtained with a specific activity of $1.4 \times 10^4$ Units/g DCW/L. The extracellular recombinant protein constituted 63% of the total extracellular protein at 12 hours post induction that decreased to 49% after 24 hours of induction (Fig 5.12). Cultivation at 20% D.O in the post induction period, thus resulted in reduced level of extracellular protein secretion by ~ 25% as indicated by a lower value of the total protein secreted after 24 hours of induction. Secretory expression of recombinant asparaginase was also lower in this case in comparison to fed-batch induced at OD$_{600}$ of 90 and maintained at a D.O of 40%. The volumetric activity and specific activity also were much lower.

**Fed-batch cultivation with lactose induction at higher cell density**

Lactose as an inducer of gene expression is an attractive alternative for cultivation in bioreactors mainly for being a cost effective inducer, it also results in the absence of higher stress response of cells associated with IPTG induction and finally lactose also lacks the toxicity associated with IPTG (non-metabolizable inducer), thus goes well with the production of recombinant proteins with therapeutic value (Gombert and Kilikian 1998; Donovan et al. 1996).

Fed-batch cultivation was carried out in a 2L computer controlled Infors AG fermentor. Concentrated nutrient feeding solution (9% yeast extract, 9% tryptone, 13.5% glycerol) was fed exponentially into the fermentor using a variable speed peristaltic pump to support a specific growth rate of 0.3 h$^{-1}$. Expression of recombinant asparaginase was induced at an OD$_{600}$ of 88.5 by a pulse addition...
of concentrated lactose solution so as to reach a final concentration of 0.4% lactose in the medium. Simultaneously the feed media was also pulsed to maintain the concentration at 0.4%. The feed was increased exponentially till the period of increasing biomass post induction. Automatic D.O control was set and maintained at 40% throughout the cultivation.

It was observed that cells continued to grow though with a declining specific growth rate even after 6 hours of induction unlike IPTG induction where growth stopped within 2 to 3 hours (Fig 5.13).

![Fig 5.13 - Time profiles of growth (OD₆₀₀, DCW), total extracellular protein and the asparaginase production in the culture supernatant during fed-batch cultivation. Arrow indicated the point of induction at an OD₆₀₀ of 88.5 with 0.4% Lactose.](image)

This resulted in achieving a relatively higher final OD₆₀₀ of 169 corresponding to a final dry cell weight of 62.5 g/L, twenty-four hours after induction. However a final volumetric yield of 5.65 X 10⁵ Units/L was obtained which is ~ 65% to that obtained with IPTG induction at higher cell density (8.7 X 10⁵ Units/L). The specific activity of 0.9 X 10⁴ Units/g DCW/L was also lower compared to value of 1.9 X 10⁴ obtained with IPTG induction. The extracellular recombinant protein
constituted 60% of the total extracellular protein at 12 hours post induction that decreased to 31% after 24 hours of induction.

Induction with 0.4% lactose thus resulted in comparatively lower low levels of extracellular asparaginase secretion in comparison to IPTG induction. The significant difference in the fraction of recombinant asparaginase present in the total protein secreted post induction suggests that lactose was not an effective inducer during the latter half of the post induction period. The flattening of the asparaginase activity profile and SDS-PAGE gel profile (Fig 5.14) starting around 10 hours post-induction demonstrating the ineffectiveness of induction. A possible reason for this is that unlike IPTG, lactose is readily consumed (Donovan et al. 1996; Neubauer and Hofmann 1994; Kotik et al. 2004) and thus not enough lactose remained in the culture to be effective as an inducer. So despite its low cost and lack of toxicity, the use of lactose as an inducer has several drawbacks, which result in low volumetric yields and difficulties in process control (Gombert and Kilikian 1998). The main inconvenience of inducing gene expression with lactose relates to the nongenetic heterogeneity of the amount of lactose permease (LacY) in the cell envelope, which varies from none to a few molecules in each cell of the culture. This phenomenon results in a long lag phase of the culture which translates to a marked decrease in volumetric yields (Chanal et al. 1998; Tolker-Nielsen et al. 1998). It is therefore critical to optimize the concentration of lactose and the strategy of lactose pulsing to obtain desired results.

The above studies in a lab scale bioreactor demonstrate the ease of scale up of this strategy for the production of recombinant asparaginase. The ability to retain good cellular health by: a) maintaining a high pre-induction specific growth rate,
b) maintaining a constant nutrient supply and c) effective inducer concentration is critical to maintain a high specific productivity, which is sustained for a long period in spite of stoppage of growth.

The success of this strategy seems to be protein specific. Clearly asparaginase being a ‘native’ protein to *E. coli* does not create problems of toxicity as is the case with many other heterologous proteins. However the ability to express the protein for long periods post-induction even after stoppage of growth is a unique feature of this system which has been accomplished primarily by effectively targeting the recombinant protein to the extracellular space.

Extracellular expression also allowed us to get the protein in fairly pure and properly folded form; all of which would aid downstream processing. Thus this step forms a critical link to the design of an integrated bioprocess strategy for asparaginase production.