Periplasmic expression of D1.3 Fab antibody in *E. coli*

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ABSTRACT

Periplasmic expression of anti-lysozyme D1.3 Fab fragment antibody was studied as a model for antibody fragment production in general since the periplasm provides a suitable oxidative environment for disulphide bond formation. However, the secretory expression has its own bottlenecks given that the translocation step puts additional burden on cells and while the synthesis rate is not tuned to the translocation capacity, it ends to the inner-membrane accumulation of premature proteins. The process-based strategy here was to decrease the fermentation temperature at the point of induction in a comparative study with conducting the whole cultivation process at a sub-optimal temperature. Both strategies ended in similar final product concentration of 26 $\mu g.mL^{-1}$ with its majority being directed to the extracellular medium. Temperature downshift improved the volumetric productivity by almost two-folds (1.7 against 0.85 $\mu g(Fab).mL^{-1}.h^{-1}$) and the periplasmic retention by 18%. Since the two strategies differed only in the temperature of the growth phase, higher antibody leakage by cultivation at low constant temperature can be explained in terms of the physiological-changes triggered by low growth temperature enhancing protein leakage. In addition, in order to examine the chance of lowering detrimental effects of induction by inducing early through logarithmic phase, preliminary studies were performed in form of shake-flask cultivations. The induction takes place early through logarithmic phase exposed cells to severe energy limitations activating genetic programs that mediate cell segregation and lysis. However, inducing at high enough biomass offers the advantage of separating the growth phase from the production phase and consequently leading higher productivity as observed for fed-batch fermentations performed in stirred-tank reactor.

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List of Abbreviations

$CFU.mL^{-1}$ – Colony forming units per millilitre

CDR – Complementarity determining region

DCW – Dry cell weight

DNA – Deoxyribose Nucleic Acid

DO – Dissolved oxygen

Dsb – Disulphide bond formation

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

Fab – Fragment antigen binding

Fc – Fragment crystallisable

Fd – Antibody heavy chain fragment consisting of the variable domain and one constant domain

HAMA – Human anti-mouse antibody

HPLC – High-pressure liquid chromatography

Ig – Immunoglobulin

IPTG – Isopropyl-β -D-1-thiogalactopyranoside

lac operon – DNA sequence regulating the expression of genes associated with the transport and metabolism of lactose in *E. coli*

OD_{xxxnm} – Optical density with xxx nm indicating the wavelength of light used for measurements

OS1 – Osmotic shock solution 1; designed to remove the outer membrane from Gram negative cells to liberate the contents of the periplasm

OS2 – Osmotic shock solution 2; designed to disrupt the inner membrane of Gram negative cells to liberate the soluble contents of the cytoplasm

PBS – Phosphate buffered saline

ppGpp - Guanosine tetraphosphate

rDNA – Recombinant DNA

SD – Standard Deviation

STR – Stirred tank reactor

TAT – Twin arginine translocase

1. CHAPTER 1 – LITERATURE REVIEW

1.1 Pharmaceutical Industry

Biotechnology is well known as a key technology in most industrial countries and it has a wide range of applications in diverse areas of medical care, food technology, agriculture, and progressively in fine chemistry (Jenzsch et al., 2006). Biotechnology uses economic exploitation of biochemical pathways, intermediate compounds, and associated final-products to facilitate the production of therapeutic products via living organisms (Burns, 1997). Commercial protein manufactures hold a significant share of the biotechnology market, and the market of biopharmaceuticals is one of the fastest growing segments of pharmaceutical industry (Burns, 1997; Azevedo et al., 2009).

Manufacture of recombinant proteins using micro-organisms dates back to the late 1970's in biopharmaceutical industry by integrating genetic and biochemical engineering (Kousar, 2008). Genetic engineering involves inserting the DNA fragments coding the gene of the desired protein into the plasmid or the genome of the host prokaryotic or eukaryotic cells. These genetically modified organisms are then fermented in bioreactors, under optimal conditions, for the production of target proteins in large amounts using biochemical engineering tools. Although for the optimization of bioprocesses, progresses have been made in both areas, greater achievements have been gained in molecular biology aspects than downstream and upstream processing (Jenzsch et al., 2006).

An important engineering challenge is the maintenance of integrity and potential viability of the molecule during downstream processing as structure of the target protein is of crucial importance for the final performance (Burns, 1997). There are several issues which further complicate the technical difficulties such as biochemical complexity of the fermentation's nature, low concentrations of the target protein at the end of fermentation (typically 10^{-1} – 10^{-6} $g.L^{-1}$), and physicochemical susceptibility of protein molecules (Burns, 1997). Downstream processing counts for up to 80% of total production costs which makes it the bottleneck of bioprocessing (Burns, 1997; Azevedo et al., 2009). For the final product to meet the end-use specifications, a series of recovery operations are conventionally employed exploiting the known chemical characteristics of the macromolecule (Burns, 1997). For example, 16 unit operations were reported as necessary for the clinical grade purification of tissue plasminogen activator (tPA) expressed in recombinant *E. coli* (Datar et al., 1993). Significant product losses arise from multiple operations due to cumulative inefficiencies and detrimental effects of multiple operations on the macromolecular integrity (Burns, 1997). Bioprocess technologies must accommodate the improvements made in molecular biology since the appeared misbalance resulted in decrease in the annual rate of approved production processes over the last decade (Jenzsch et al., 2006).

In therapeutic proteins manufacturing, the key criterions to the improvement of efficiency and performance of bioprocesses are high cell concentration and high quality product yield. To enhance the micro-organism growth, both genetic-based solutions and medium developments have been employed.

An example of genetic modifications is adapting the gene expression rate with metabolic activity of the cells to make a balance between production of recombinant and cellular proteins which results in a steady and prolonged protein synthesis (Cserjan-Puschmann et al., 2002). In a study by Chou et al. (1994), an *E. coli* mutant strain with a modified glucose uptake rate was constructed to avoid the formation of acetate, the primary inhibitor of cell growth and recombinant protein expression, which is normally observed in high levels in dense cultures. The slightly modified glucose uptake prevented acetate from accumulation to toxic levels by delaying the onset of its production which led to a 50% level of improvement in both biomass concentration and recombinant protein productivity.

Medium developments involve modifying nutrients compositions and types of amino acids used in growth medium resulting in an improved biomass concentration. Towards this approach, attempts have been made to prevent the production of acetate by controlling the feed rate of carbon source in a stirred tank reactor (STR) (Kousar, 2008) or to improve the protein yield by replacing a slower metabolising carbon source such as glycerol (Jenzsch et al., 2006) and fructose (Aristidou et al., 1999).

1.2 Structure, Characteristics, and Generation of Antibody Molecules

1.2.1 Immunoglobulins

Antibody, a member of immunoglobulins family, is a plasma protein (Janson and Rydén, 1998). Immunoglobulins (Ig), no matter of specificity, can all be described as Y-shaped

symmetrical molecules, consisting of four polypeptide chains: two identical glycosylated heavy chains (H) of M_r ~ 50 – 75 KDa and two identical unglycosylated light chains (L) of M_r ~ 25 KDa (Goding, 1986; Janson and Rydén, 1998; Vaughan et al., 1998; Conroy et al., 2009). Disulfide bonds pair the heavy chains together and link each light chain to a heavy chain. There are two types of light chains, κ and λ, whereas five types of heavy chains exist (Goding, 1986; Janson and Rydén, 1998; Conroy et al., 2009).

Immunoglobulins can be classified according to their heavy chains as five major classes of IgG, IgA, IgM, IgD, and IgE which possess γ, α, μ, σ, and ε heavy chains, respectively (Goding, 1986; Janson and Rydén, 1998; Conroy et al., 2009). Individual classes of immunoglobulins have distinctive structural and biological properties.

IgG is the most abundant class of immunoglobulins in serums, the most widely used antibody for the construction of immune library, and the main choice for immunoelectrophoresis (Goding, 1986; Janson and Rydén, 1998; Conroy et al. 2009).

Heavy and light chains are composed of a series of repeating segments of about 110 amino acids, called *homology units* (Goding, 1986; Janson and Rydén, 1998). Each homology unit is folded into a compact globular structure known as *Ig domain*, which contains large amounts of β-pleated sheets and is rather resistant to proteolysis, in contrast to short sequences linking the domains (Goding, 1986; Janson and Rydén, 1998). The term *variable domain* refers to the N-terminal homology units that their amino acid sequences vary markedly between molecules. The sequences of C-terminal

homology units are relatively conserved and are therefore known as *constant domains* (Goding, 1986).

Heavy chain has one variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3) while light chain has two successive domains: one variable domain (V_L) and one constant domain (C_L). The diversity in variable domains is restricted to three *hypervariable regions* within each domain. The variable domains of heavy and light chains are folded in a way that brings the hypervariable regions together to form loops. These *hypervariable loops* comprise the antigen-combining site known as *complementarity determining regions* (CDRs). Both light and heavy chains are involved in determining the specificity of antigen-combining sites. The remaining amino acid residues of variable domain form the *framework region*, which acts as a scaffold to support CDRs (Goding 1986; Alberts, 2002).

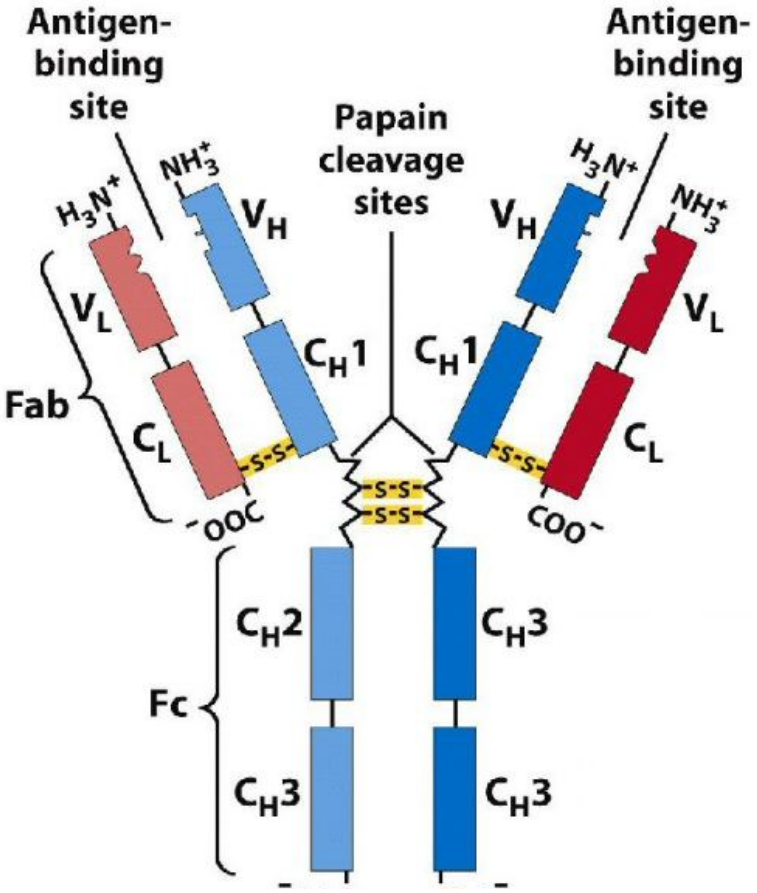

Figure 1.1 Schematic representation of full-length IgG molecule. The molecule is composed of two identical heavy chains and two identical light chains. Heavy chain consists of one variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3). Light chain has two domains: one variable domain (V_L) and one constant domain (C_L). Disulphide bonds link each heavy chain to a light chain and the two heavy chains together. (Nelson et al., 2008)

1.2.2 IgG Fragmentation and Generation of Fab Antibody Fragment

IgG molecule consists of two identical halves. As mentioned earlier, inter-domain linkers are susceptible to proteolytic attack, therefore, the controlled proteolysis of immunoglobulins may lead to fragmentation at joints. Proteolysis reaction of IgG by the enzyme papain is of major importance as the cleavage at the hinge leads to the

generation of two identical Fab fragments and one Fc fragment (Andersen and Reilly, 2004; Goding, 1986). Fab fragment contains one intact light chain disulfide-bonded to Fd (a heavy chain fragment consisting of V_H and C_H1 domains). Fc is a dimer of the remaining portion of the two heavy chains, C_H2 and C_H3, which is a homogeneous fragment with no antigen-binding activity and modulates the immune system response (Goding, 1986). The papain cleavage site, Fab and Fc fragments are shown in Figure 1.1.

1.2.3 Development in Antibody Generation

B lymphocytes carry specific receptors that recognize the antigenic-determinant of antigens and react by the production of antibodies with the required specificity. The term *monoclonal antibody* refers to the class of antibodies, produced by B cells against one specific antigen, possessing the same chemical structures. Antibodies produced against an antigen with multi antigenic determinants are called monospecific polyclonal antibodies. The term *monospecific* refers to the class of antibodies that produce against the same antigen while *polyspecific* is used where the immune response is against several antigens. (Janson and Rydén, 1998)

Unmodified murine antibodies/antibody fragments were the first generation of monoclonal-based pharmaceutical products (Walsh, 2003). Drawbacks accompanied by administration of monoclonal antibodies include eliciting human anti-mouse antibody (HAMA) response and the inability to trigger the human immune system effector functions such as activation of complement (Leong et al., 2001; Walsh, 2003). These difficulties have persuaded integration of engineering approaches using hybridoma

technology to produce chimeric antibodies with the capability of performing human effector functions and reduced immunogenicity by fusing the gene sequence coding for the constant domains of a human antibody to a nucleotide sequence coding for the murine CDR loops (Vaughan et al., 1998; Walsh, 2003). Development of humanized antibodies accomplished using an even more extensive engineering approach which involves grafting a murine antigen binding site (CDR loops) onto a human antibody framework (Hudson, 1998; Leong et al., 2001; Walsh, 2003). The resultant antibodies elicit reduced immunogenicity in humans even compare to chimeric antibodies and exhibit almost identical serum half lives to native human antibodies (Walsh, 2003). Over the last few years, a number of chimeric and humanized antibodies have been medically approved (Walsh, 2003). However, using recombinant proteins as an alternative to monoclonal and polyclonal antibodies offers several advantages including the possibility for novel and rare functionalities, the increased rate of antibody generation, and the possibility to modify the affinity and specificity of antibody molecules (Alcocer et al., 2000).

1.2.4 Application of Antibodies as Cancer Therapeutics

The ability of antibodies to specifically bind to cell surface antigens expressed on tumor cells made them an attractive option for cancer therapeutics (Kubetzko et al., 2006). Both direct delivery of cytotoxic agents and indirect strategies have been investigated using unarmed antibodies, fusion proteins, or chemical immunoconjugates. Indirect strategies include blocking the cellular growth factor or their receptors, complement-

dependent cytotoxicity or induction of apoptosis by receptor engagement (Kubetzko et al., 2006; Trikha et al., 2002).

Mode of action of the therapeutic molecule determines the requirements that the pharmacokinetics properties need to meet. For example, in case of cytotoxic action, restriction of the action to the tumor site is quite important and the activity in normal tissues has to be limited both in magnitude and duration. While cytotoxicity is not the desired action, high concentration of therapeutics is required at the tumor site and accumulation at normal tissues would be less harmful. As the molecular properties of the antibody dictate its *in vivo* behaviour, the understanding of how changes in molecular format affect the pharmacokinetic behaviour is essential. For example, in case of tumor targeting, the critical determinants are specificity, affinity, valency, stability, surface charge, and molecular size (Kubetzko et al., 2006). A number of investigations have reported that despite the fact that affinity increases tumor localization and retention, there is an upper limit in affinity beyond which no further improvements can be achieved due to a number of limiting factors in both *loading phase* and *retention phase* (Adams et al., 2001; Kubetzko et al., 2006).

Antibody fragments including Fabs, Fab'2s, and single chain Fv forms may be the more suitable therapeutic choice because of their smaller size for some of those applications where there is no requirement for Fc mediated effector functions such as tumor penetration and imaging (Andersen and Reilly, 2004). However, these fragments possess shorter circulating half life compare to full length antibodies (Andersen and Reilly, 2004; Leong et al., 2001).

1.2.5 Functional Improvements of Antibody Fragments

Several attempts have been made to conquer the short circulating half life of truncated antibodies which is a limiting factor for several therapeutic applications. Site-specific attachment of polyethylene glycol (PEG) moiety has been examined by Chapman et al. (1999) and resulted in a circulating half-life equivalent to 80% of that from its parent IgG molecule with the full retention of the antigen binding affinity. In addition, modulation of circulating half-life by the choice of size and structure of PEG attached has been studied (Leong et al., 2001). Attachment of an albumin-binding peptide to the C terminus of a Fab light chain ended to a 26- to 37-fold increase in circulating half-life in either mice or rabbits (Dennis et al., 2002). The progresses that have been made to increase the circulating half-life allows less frequent patient dosing and can extend the therapeutic usefulness of antibody fragments (Andersen and Reilly, 2004).

Although these antibody fragments can be generated by treating the mammalian-produced full-length antibodies with pepsin or papain, *E. coli* still remains as the main workhorse for the production of recombinant antibody fragments (Andersen and Reilly, 2004). Improvements in expression technologies needs to be speeded up to support the increasing demands for clinical applications of recombinant antibodies and antibody fragments.

1.3 *Escherichia coli* for Recombinant Protein Production

Escherichia coli (*E. coli*) is the most widely used host for the production of proteins and non-protein biomolecular products like amino acids, primary and secondary metabolites as it has been well characterized in terms of genetics, molecular biology, and expression systems (Lee, 1996; Makrides, 1996; Choi et al., 2006; Swartz, 2001). *E. coli* cannot be used for the production of some large complex eukaryotic proteins containing multiple disulphide bonds or proteins that need post-translational modifications (Lee, 1996; Makrides, 1996; Swartz, 2001; Choi and Lee, 2004). In the case of these products, there is a possibility of the formation of insoluble, biologically inactive inclusion bodies, which can only be recovered as biological active forms through complicated and costly denaturation and refolding processes (Lee, 1996; Choi and Lee, 2004; Choi et al., 2006). Although successful productions of a number of economically sensitive products as insulin and bovine growth hormone using *E. coli* as the host suggests the potential economic feasibility, *in vitro* protein folding process is industrially undesirable to implement (Swartz, 2001). However, recent progress in disulfide bond formation and protein translocation has made the possibility to design recombinant *E. coli* strains for these products (Lee, 1996; Makrides, 1996; Swartz, 2001).

Recombinant protein expression can be targeted to any of the three compartments to which *E. coli* is divided to by the inner and outer membranes: cytoplasm; periplasm and the extracellular milieu. A balancing needs to be made between advantages and disadvantages of each compartment to determine its suitability for targeting recombinant proteins (Choi et al., 2006; Pierce et al., 2002). The main issues concerned

with the selection of a recombinant microorganism and its form of protein expression from cultivation prospective includes: specific and volumetric productivities; concentration and percentage of the target protein in the feedstream before and after purification and total culture time. Those issues related to the downstream processing are the volume of process streams and the number and efficiency of purification steps required (Pierce et al., 2002). There are several factors affecting the choice of an expression system for high-level production of recombinant proteins, including cell growth characteristics, the end-use of target protein, expression levels, and intra- or extra-cellular production. Each expression system has specific expenses in terms of process design and economic considerations (Choi et al., 2006).

Details on targeting recombinant protein expression to these three compartments have been covered in this section.

1.3.1 Cytoplasmic Protein Expression

Intracellular expression benefits from the protein retention within the cell, which can reduce the processing volume. However, a higher contaminant protein would be loaded in subsequent processing stages (Pierce et al., 2002). High-level production of heterologous proteins in cytoplasm may lead to the formation of inclusion bodies, which results from accumulation of folding intermediates rather than unfolded or native proteins (Choi et al., 2006). No general role has been detected for the recognition of those proteins that have tendency to make inclusion bodies. In a study by Kane et al. (1988), a number of heterologous proteins were examined to study the effect of

properties and conditions of *E. coli* cytoplasmic expression system on the formation of inclusion bodies. No particular relationship was observed between inclusion bodies formation and protein origin, utilised promoter or hydrophobicity of target protein.

Several strategies have been developed to aid correct folding of proteins in cytoplasm including culturing at low temperatures (Schein, 1989), lowering the protein expression rate (Bowden and Georgiou, 1990), protein fusion with highly soluble polypeptides (Uhlen et al., 1990; Murby et al., 1996), and co-expression of molecular chaperons (Wall et al., 1995; Bessette et al., 1999). The increased level of production that achieved for several monomeric and multimeric proteins using these strategies appeared to be protein-specific. In addition, the high content of total cellular proteins in cytoplasm makes the purification of soluble target proteins difficult.

Interestingly, inclusion bodies have several advantages compared to soluble proteins including the higher accumulation in cytoplasm, high protein titre using HCDC, and increased protein yields as they are proteolysis-resistant. In addition, they can be isolated in a highly concentrated state by a simple centrifugation step. However, the necessity for sophisticated and costly denaturation and refolding processes to recover biologically active proteins from inclusion bodies makes cytoplasm an unattractive location for targeting protein expression (Choi et al., 2006). Additionally, the hydrophobic regions of theses soluble refolded proteins may interact with each other resulting in the formation of aggregates which usually reduce the final yields considerably (Choi and Lee, 2004).

1.3.2 Extracellular Protein Expression

Protein secretion to the extracellular medium of microorganisms can be obtained either by fusion proteins or development of leaky mutants in gram negative bacteria including *E. coli* or by active secretion in gram positive bacteria (Pierce et al., 2002). Researchers prefer to use those microorganisms that lack the outer membrane (gram positive bacteria) or those equipped with good excretory mechanisms such as yeasts (Choi et al., 2006). A purer initial protein might be obtained by fully secretion since the protein is already released from the cell and fewer unit operations are involved in its recovery with the culture broth being clarified only before chromatography. However, the processing volume can be very large and there is a chance of protein denaturation at air liquid interfaces in the bioreactor (Pierce et al., 2002).

1.3.3 Periplasmic Protein Expression

To overcome the inherent problems associated with the cytosolic expression, many researchers have switched to targeting proteins to the periplasmic space (Choi et al., 2006). Furthermore, from a bioprocessing point of view, to facilitate the purification of foreign proteins, the best option other than secretion to the extracellular milieu is to target protein to the periplasm (Want et al., 2009). Periplasmic expression capitalises on attributes of the other two expression systems by allowing selective release of the protein into a smaller process volume by dividing it into separate compartments and a lower contaminant protein loading compared to the cytoplasm (Pierce et al., 2002). Targeting protein to the periplasm offers several advantages: protein degradation is of

less concern in periplasm than cytoplasm owing to the significantly lower levels of proteolysis; simpler purification due to a lower protein content compared to the cytoplasm; it provides the oxidative environment required to aid the correct protein folding; offers the possibility to conduct *in vivo* activity assays due to greater access of the targeted protein to the substrate (Murby et al., 1996; Choi et al., 2006). In addition to the clarification of culture broth prior to chromatography, cell harvesting is required in both periplasmic and cytoplasmic expression systems (Pierce et al., 2002).

Generally, proteins found in periplasm or outer membrane are synthesized in the cytoplasm as premature proteins containing short specific amino acid sequence known as signal sequence with which they can be exported outside the cytoplasm (Choi and Lee, 2004). Two distinct pathways exist for the periplasmic localisation of proteins in *E. coli* (DeLisa et al., 2003). The majority of proteins are translocated in an unfolded form via the general secretory (Sec) pathway. Proteins incompatible with Sec pathway, such as those requiring association of subunits or integration of cofactors prior to secretion for which they need to be in folded form, are transported across the cytoplasm via a completely different pathway, *Twin* arginin translocation (Tat) pathway. Disulphide bond formation is a crucial post-translational modification and functions to stabilise the three-dimensional structure of a protein by reducing the chain entropy since the cysteines that are linked by disulphide bonds can take on far fewer conformations (Nakamoto and Bardwell, 2004). Dsb (disulphide bond formation) proteins include DsbA and DsbB which contribute to disulphide bond formation and DsbC and DsbD that are involved in disulphide bond isomerisation in *E. coli* periplasm. For the polypeptide to retain in unfolded state through localisation, for post-translational system it carries

out by the attachment of anti-folding agents and in the co-translational pathway secretion begins shortly after the initiation of biosynthesis (Jalalirad, 2010).

Nevertheless, there are a number of problems that hinder efficient production of heterologous proteins targeted to the *E. coli* periplasm (Choi and Lee, 2004). In the case of Fab fragments, this obstructs the achievement of fully functioning biomass (Want et al., 2009). Balagurunathan and Jayara (2008) inferred that the sole cause of growth impairment observed upon induction of secretory expression of the recombinant streptokinase in *E. coli* was the presence of signal peptide since intracellular expression of the same recombinant protein did not cause any disruption. To overcome the obstacles of secretory protein expression, several individual biological strategies have been examined by generally addressing one particular step of the complex process of protein secretion at a time. This may not be always beneficial for improving productivity since each process involved in protein secretion is modulated by several other factors and the coordinated function of several processes needs to be taken into consideration. Instead, identifying the biological constraints and exploitation of process strategies may affect many of these individual processes and involve the interactions in between. Balagurunathan and Jayara (2008) improved the overall productivity of the recombinant secretory system by lowering the cultivation temperature, where accumulation of recombinant protein with signal peptide in inner membrane triggered the expression of an inner membrane protease, which subsequently led to growth impairment and degradation of synthesised protein, hence lowering the productivity. Garcia-Arrazola et al. (2005) have addressed the influence of glycerol oscillations on Fab' yield, product localisation, and product loss during centrifugation. They performed

two separate repeated batch fermentations, one with a pH-stat feeding strategy to conduct a constant minimum concentration of the carbon source by coupling the pH control with C-source addition. A research by Want et al. (2009) has also been undertaken to investigate the effect of induction point on the product yield of anti-lysozyme Fab fragment, biomass concentration, and cell physiological state.

Cell envelop consists of a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane. Methods used for cell disruption to release the intracellular contents can be classified as mechanical and non-mechanical categories. Non-mechanical methods which result in more specific and gentler destruction (Geciova et al., 2002) include physical, chemical, and enzymatic methods. Osmotic shock is a well-established method and seems to be promising for large-scale operation since no cost-inhibitory or detrimental agent is used. In osmotic shock procedure, cells shrink following the suspension into a sucrose solution of high osmotic shock strength which is supplemented with EDTA to release the lipopolysaccharide, the permeability barrier, from the outer membrane by chelation of the magnesium ions (Chen et al., 2004). That is where the liberation of the periplasmic contents occurs. Then the cells are exposed to osmotic shock gradient by suspension into a sucrose-deficient solution to cause an influx of water to the periplasm resulting in swell and burst of the periplasm and release of cytoplasmic contents.

Once the expression system has been developed, the high cell density culture (HCDC) technique would be used to increase the protein product titre. Various stages of recombinant protein expression system including transcription, translation, secretion,

and proteolytic degradation can be affected by feed composition, feeding strategy, and physical parameters of cultivation such as temperature, pH, and dissolved oxygen (DO).

1.4High Cell Density Culture (HCDC)

There are some pharmaceutical proteins that have been manufactured on a small scale, typically a few tens of kilograms per year and have made profits, but when it comes to a competing drug or a specific product being made with more than a company, reduction in production costs becomes more significant. Thus, the primary goal in fermentation is to make a cost-effective production. As most proteins are intracellulary accumulated in recombinant *E. coli*, a higher productivity can be gained by increasing the cell density and the specific productivity, which is the product formed per unit mass per unit time. Development of high cell density culture for recombinant protein production in *E. coli* allows higher productivity by targeting the specific productivity and has the along-side advantages of reduced culture volume, enhanced DSP, reduced wastewater, and lower production and capital costs (Mori et al., 1979; Lee, 1996; Choi et al., 2006). Fed-batch fermentation is the frequently used method for the achievement of HCDC for recombinant *E. coli*. HCDC has been successfully achieved in fed-batch mode of operation by sustaining the maximum specific growth rate that had been used for inoculation using different nutrient feeding regimes. Cell concentrations of greater than 50 $gDCW.L^{-1}$ have been obtained for fed-batch fermentations of recombinant and non-recombinant *E. coli* (Lee et al., 1996).

Several attempts have been made to determine the maximum cell concentration in a tightly packed fermenter. Markl et al. (1993) found out that the maximum theoretical cell concentration of *E. coli* in culture broths containing tightly packed cells of 3 μm long and 1 μm diameter is within a range of 160-200 gDCW.L^{-1}. Throughout the calculation, they figured out that the culture medium only makes up 25% of the culture, the dry cell weight is about 20-25% of the wet cell weight, and that the cells are slightly denser than water. The very high cell concentration of 174 gDCW.L^{-1} that they achieved from cultivating *E. coli* W3110 cells on a glycerol-based mineral medium in a dialysis reactor with two chambers was within the theoretical range. There is also a report on the achievement of a high cell density of 175.4 gDCW.L^{-1} for recombinant *E. coli* XL 1-Blue producing poly(3-hydroxybutyrate) (Lee and Chang, 1994). Mori et al. (1979) observed a sharp increase in the viscosity of the culture broth as the cell concentration exceeded 200 gDCW .L^{-1} until the point that it almost lost its fluidity at the concentration of 220 gDCW.L^{-1}. The theoretical maximum achievable cell density of 200 gDCW.L^{-1} is supported by this finding, in addition to the close match with the highest cell densities achieved.

HCDC technique has led to efficient, high-level production of protein and non-protein products expressed in *E. coli* (Lee, 1996). There are, however, several drawbacks to using HCDC techniques such as substrate inhibition, limited availability of dissolved oxygen at high cell concentrations, accumulation of carbon dioxide to inhibitory levels, reduced mixing efficiency, and heat generation (Lee, 1996; Choi et al., 2006). In order to solve these problems, a variety of techniques have been developed targeting different parameters or aspects of recombinant protein production such as dissolved oxygen

regulation, use of different host strains, different promoters to regulate level of expression, or an appropriate feeding strategy (Choi et al., 2006). Throughout this section, main problems associated with HCDC of *E. coli* and their respective proposed solutions have been discussed in more details.

1.4.1 Physical Parameters

1.4.1.1 Temperature of Fermentation

Temperature plays an important role in the solubility of expressed proteins, the culture productivity (Thiry and Cingolani, 2002), and controlling the cell metabolism (Lee, 1996). Lowering the culturing temperature from 37°C to the range of 26-30°C decreases the formation of toxic by-products and generation of metabolic heat by reducing the nutrient uptake and growth rate (Lee, 1996). Higher cell densities can also be achieved without the need for pure oxygen since at lower temperatures, the cellular oxygen demand is lesser (Lee, 1996). There is a report on the achievement of higher levels of soluble proteins by culturing at the temperature of 30°C compared to processing at 37°C which led to the formation of inclusion bodies (Jensen and Carlsen, 1990). A fourfold increase was observed in the yield of galactose oxidase expressed in recombinant yeast *Pichiapastoris* by cultivating at 25°C instead of 30°C in shake flask (Whittaker and Whittaker, 2000). Hence culturing at lower temperatures has attracted attention in order to gain HCDC (Lee, 1996).

1.4.1.2 pH Culture

pH needs to be selected as a value for the medium to maintains the stability of the protein being expressed. The level of importance for optimization of the pH medium depends on the host strain and target protein, for example it is highly crucial for protein expression in yeasts because they can grow in a wide range of pH (Thiry and Cingolani, 2002). Cultivating *Phichiapastoris* which cannot grow at pHs below 2.2 is preferential at lower pHs since it facilitates the prevention of degradation by proteases (Thiry and Cingolani, 2002). However, it was essential to raise the pH from 4.5 in the growth phase to 6 for the methanol induction phase to maintain stability of the target protein galactose expressed in *Phichiapastoris* as it is not stable at lower pH for prolonged time (Whittaker and Whittaker, 2000).

1.4.1.3 Dissolved Oxygen of Culture

The point that carbon substrate becomes a growth limiting factor and the culture needs to be fed can be indicated by dissolved oxygen of the culture (DO) (Mori et al., 1979). Oxygen itself is likely to become a limiting factor in HCDC due to its low solubility. The saturated concentration of oxygen in water is 7 mg l^{-1} at the temperature of 25°C and the pressure of 1 atm but the aeration and stirring rate can be used to control the oxygen supply (Lee, 1996). Dissolved oxygen often acts as a limiting factor at high growth rates (Lee, 1996; Thiry and Cingolani, 2002).

Supplementing air with oxygen or use of pure oxygen is advantageous to avoid oxygen limitations (Lee, 1996; Thiry and Cingolani, 2002). However, use of oxygen-enriched air or pure oxygen is restricted by its cost, especially for large-scale production since a larger quantity is required (Lee, 1996).

Oxygen limitation can also be prevented by lowering the growth rate which reduces the cellular oxygen demand. Riesenberg et al. (1991) observed a rise in the expression rate of the recombinant interferon α-1 in *E. coli* at lower growth rates and the maximum productivity was achieved at the specific growth rate of 0.11 h^{-1}. Also higher biomass concentrations achieved at lower growth rates in chemostat cultures of *Aerobacteraerogenes* on a minimal defined medium with glucose, $(NH_4)_2SO_4$(N), K^+, Mg^{2+}, SO_4^{2-}, and PO_4^{3-} as the limiting nutrients in turn (Dean and Rogers, 1967). It is also known that for enzyme production, there is a maximum to reach in terms of the expression rate as a function of growth rate. Additionally, by culturing the cells at a lower specific growth rate, the rate of heat dissipation can be diminished as the major sources of heat generation includes mechanical energy of agitation and metabolic heat generated by cells (Lee, 1996). Therefore, it is required to develop expression process strategies that support cultivations at reduced growth rates; below the threshold value triggering the formation of inhibitory by-products and optimal for expression of the desired product. Optimal growth rate needs to be determined in terms of strain and operational conditions.

The other strategy to increase the solubility of oxygen is to apply pressure, however, it ends to formation of more carbon dioxide and may enhance the detrimental effects of

high partial pressure of carbon dioxide (>0.3 atm), including reduction in growth rate and increase in acetate formation (Lee, 1996; Thiry and Cingolani, 2002).

In a study by Chauhan et al. (2001), the optimal point of induction was found as the moment where oxygen transfer was maximum and the dissolved oxygen concentration was nought.

1.4.2 Type of Medium

Growth media may be categorized into three types of defined, semi-defined and complex medium. Although complex media is cheap, easy to prepare and provides faster growth, its analysis is difficult and batches may vary from one to the other (Thiry and Cingolani, 2002). By using defined media, a well-defined and reproducible cultivating condition can be obtained as well as a simpler purification since there is none of those proteins or other contaminating cellular components that are used in the composition of complex media. *Defined minimal medium* is referred to a defined medium only containing the nutrients necessary for growth, which is typically a simple carbon such as glucose, inorganic salts, trace elements, or individual forms of vitamins or amino acids (Yee and Blanch, 1993). In general, defined media is the option for the achievement of high cell density, especially when it is desired to control the specific growth rate, as the concentrations of nutrients are known and can be controlled throughout the fermentation (Yee and Blanch, 1993; Lee, 1996). However, it is sometimes necessary to design a semi-defined or complex media to boost the product formation.

A balanced optimized medium needs to be developed to achieve higher growth rates, growth yields, and cell densities (Yee and Blanch, 1993). The feeding solution is required to contain all the nutrients essential for supporting cell growth (Lee, 1996). A combination of cellular composition and previously used media along with a random selection of medium constituents and their forms and concentrations are usually used to provide satisfactory growth in traditional shake-flask cultivations and batch fermentations. However, development of an optimized medium for a balanced growth requires nutrient studies.

Early media optimizations were conducted in a sequence of shake flask experiments by changing the concentration or nature of the elements and monitoring the consequent change in growth rate and/or cell density. This is a simple procedure, even though several drawbacks have restricted its application including the requirement for considerable work and time and the probable changes that might occur in operational conditions during growth in shake flasks which complicates the result analyses (Yee and Blanch, 1993). A faster and more reliable alternative is pulse injection technique that facilitates determination of qualitative aspects of an optimized medium in a continuous culture or a chemostat. To determine the qualitative aspects, all the components either necessary for or inhibiting the cell growth needs to be identified. While the components necessary for growth have been identified, a series of single nutrient-limited growth in chemostats must be performed to determine the yield coefficient of each element. Subsequently, an optimized balanced growth medium can be formulated, only limited in one substrate. Development of an optimized, glucose-limited defined minimal medium resulted in a maximum specific growth rate of 0.87 h^{-1} in chemostat cultivation of *E. coli*

for the production of the recombinant protein, rat anionic trypsin (Yee and Blanch, 1993).

Generally, the cellular growth in *E. coli* can be inhibited while the following nutrients are present above certain concentrations: glucose (50 g l^{-1}); ammonia (3 g l^{-1}); iron (1.15 g l^{-1}); magnesium (8.7 g l^{-1}); phosphorous (10 g l^{-1}); zinc (0.038 g l^{-1}). Thus, the initial concentrations need to be defined below the inhibitory threshold and additions can be made during the cultivation while required to maintain appropriate growth rates. It is preferential to use non-carbon and non-nitrogen sources instead; in sufficient amounts to make the simplest possible feed solution (Lee, 1996). Riesenberg et al. (1991) developed an especially designed glucose/mineral salt medium and a fed-batch cultivation method by controlling the growth rate and the pressure of oxygen. This economy procedure, indicated by high coefficient yields, has resulted in a high biomass concentration of 110 gDCW.L^{-1} and suppression of inhibitory by-products generation. One of the most frequently used nutrients as nitrogen source is ammonium hydroxide, as it can also be used to adjust the pH of the culture. Medium optimization is a labour-intensive process as it requires processes based on trial-and-error experiments to test several nutrient combinations.

1.4.3 Feeding Strategies

Achievement of a successful HCDC with high productivity depends on feeding strategy as it affects maximum attainable cell concentrations, specific productivity of recombinant proteins, and by-products formation (Lee, 1996; Choi et al., 2006). Several HCDCs have

been carried out using simple feeding strategies including constant rate feeding (Wong et al., 1998), stepwise feeding, and exponential feeding (Yoon et al., 1994; Qiu et al., 1998; Babu et al., 2000).

In constant rate feeding where the concentrated nutrients are fed into the bioreactor at predetermined concentrations, the specific growth rate decreases as the cell population and culture volume increase and the rate of rise in cell concentration decreases over time. By increasing the feed rate gradually (stepwise addition), more nutrients are supplied at higher cell concentrations boosting cell growth. Exponential growth of cells can be achieved while feed rate of the growth limiting substrate is increased proportionally with cell growth (Lee, 1996).

Exponential feeding allows cell growth at specific constant rates and by maintaining specific growth rate below the critical value of acetate production, acetic acid formation can be diminished (Choi et al., 2006; Lee, 1996). To estimate the feeding rate suitable for achieving exponential growth with constant specific growth rate, a simple mass balance can be used based on the assumption of constant cell yield on substrate. Exponential feeding is a simple efficient method by which successful HCDCs for several non-recombinant and recombinant *E. coli* strains have been achieved (Lee, 1996). The specific growth rate usually needs to be maintained in the range of 0.1 and 0.3 h^{-1} to prevent acetate generation (Lee, 1996). The values of 0.35 and 0.2 h^{-1} have been reported as the inhibitory threshold for specific growth rate of *E. coli* cells grew on a minimal and a complex medium, respectively (Choi et al., 2006). Cell concentrations of

128 g(DCW) l^{-1} and 148 g(DCW) l^{-1} have been attained by exponential feeding during glucose- and glycerol-limited growth respectively (Korz et al., 1995).

More complicated feeding methods have also been developed making use of a feedback control scheme like coupling of feeding with measurement of various physical parameters, such as DO, pH, microbial heat, and CO_2 evolution rate (CER) (Lee, 1996). pH and DO increase sharply by substrate depletion. The former one is due to an increase in the accumulation of ammonium ions excreted by cells following carbon exhaustion. Since the concentration of microorganism achieved for fed-batch culture is usually greater than 10 g(DCW) l^{-1}, it is likely that oxygen becomes a growth-limiting factor. By using a DO-stat, stable operation can be achieved through supplying constant dissolved oxygen concentration during the cultivation. DO-stat facilitates the use of pure oxygen or oxygen-enriched air by lowering the chances of toxicity due to high DO (Mori et al., 1979). Whenever a rise in DO is observed, automatic addition of a predetermined concentration of nutrient can be made in order to maintain the substrate concentration within a desired range (Lee, 1996). Since the evolution rate of carbon dioxide during cell growth is roughly proportional to the consumption rate of carbon substrate, nutrient feeding can be controlled using the concentrations of carbon dioxide from the gas outlet (Lee, 1996).

In defined media, nutrient depletion has a considerably higher effect on DO-stat than pH-stat. Complex medium initially contains complex carbon-nitrogen sources in addition to carbohydrates. Thus, the cells continue to use complex nutrients upon carbon

substrate depletion and no significant changes can occur in DO. Therefore, in case of semi-defined or complex medium, pH-stat is a more appropriate option (Lee, 1996).

Specific growth rate (μ) at the time of induction was found to have significant effect on production rate and protein accumulation (Sandén et al., 2003). Formation of ppGpp, the main factor of the stringent regulation response, occurred at high and low μ which was ascribed to the limitation in precursors and carbon source, respectively. Higher levels of ppGpp formation and acetate accumulation at high μ indicate the higher metabolic burden on cells at higher production rates. Although exponential feeding usually maintains a rather constant specific growth rate, it cannot control the changes that occur in the specific growth rate during the culture (Lee, 1996). Several methods have been developed to maintain $\mu<\mu_{inh}$ during fed-batch culture: increased glucose feeding under computer control by calculating the feeding rate from the amount of glucose fed up to that point (achieved a maximum biomass concentration of 53 $gDCW.L^{-1}$) (Paalme et al., 1990); a pO_2 (oxygen partial pressure) control loop by varying glucose feeding along with a μ control loop by variations of agitation speed (attained a cell concentration of 110 $g(DCW)\ l^{-1}$) (Riesenberg et al., 1991).

Specific growth rates have been more accurately controlled using recent developments in on-line sensors and control strategies: an automated control method using a laser turbidimeter to continuously measure the cell concentration and culture volume (Yamane et al., 1992); a direct feedback control method by measuring on-line the concentration of the growth limiting substrate and automated feeding to the pre-set value (Kleman et al., 1991); a feedforward-feedback control type of strategy with

changes in pH and μ as the inputs to a FNN (fuzzy neural network) to calculate exponential feeding rate (Ye et al., 1994).

1.4.4 Acetate Formation

The key behind the goal of an economic and efficient recombinant expression micro-organism system is to achieve high cell density and high level of gene expression. Glucose is the substantial nutritional supplement as it is inexpensive and rapidly metabolisable carbon and energy source (Luli and Strohl, 1990; Aristidou et al., 1999). One of the main factors inhibiting the high volumetric recombinant protein productivity (the product formed per unit volume per unit time) in *E. coli* is the conversion of glucose to toxic by-products, mainly acetate, either through overflow metabolism or mixed acid formation (Luli and Strohl, 1990; Chou et al., 1994; Aristidou et al., 1999; Kousar, 2008). Overflow metabolism is caused by an imbalance between the carbon metabolism and respiration (Elmansi and Holms, 1989; Kleman et al., 1991). In this condition, the carbon influx to the central metabolic pathway exceeds the needs for biosynthesis, which is mediated by the excretion of acetate and other organic by-products. However, mixed acid formation occurs in oxygen limitation conditions (Kousar, 2008).

Several studies have been carried out on the effect of fermentation conditions on acetate formation such as designing optimum feeding strategies to maintain low levels of residual glucose or diverting excessive carbon fluxes to a less detrimental product as well as the sensitivity of different *E. coli* strains to overflow metabolism (Luli and Strohl, 1990; Aristidou et al., 1999). In case of optimization of strategies for nutrient feeding,

very elaborate techniques would be required to maintain glucose concentration below the level that is the onset of switch over from normal coupled respiration to acidogenesis (1-2 $g.L^{-1}$) (Aristidou et al., 1999). Therefore, the idea of replacing glucose with a comparable substrate in terms of accessibility and cost which generates nought or lower levels of toxic side-products seems beneficial. It has been demonstrated that by replacing glucose with a slowly metabolising carbon source such as glycerol and fructose, protein yield can be improved (Aristidou et al., 1999).

One of the main factors that needs to be taken into considerations while optimising the fermentation conditions using IPTG as the inducing agent is the glucose repression. Maintaining the limiting concentrations of glucose during the induction phase was found to be more important to the attainment of high rate of heterologous protein production than inducing at lower specific growth rates (Kilikian et al., 2000). Glucose repression can be avoided by fed-batch feeding of glucose during the induction phase or by replacing glucose with a non-repressing carbon molecule after induction.

Glycerol can be used to prevent acetate formation because glycerol transfer rate is lower than glucose, thus the carbon flux through glycolysis can be reduced (Lee, 1996; Aristidou et al., 1999). A feeding strategy was developed for HCDC of *E. coli* in a defined medium by exponential feeding of glucose or glycerol as the carbon source and maintenance of specific growth rate below the inhibitory threshold (Korz et al., 1995). Final cell concentration achieved was higher while glycerol was used as the carbon source (148 in comparison with 128 $gDCW.L^{-1}$) because glucose cannot be used in high concentrations as glycerol due to its low solubility and because the cell growth at lower

rates was supported by glycerol. However, glycerol is more expensive (Lee, 1996; Aristidou et al., 1999) and cells grow more slowly on glycerol (Lee, 1996) which on the other hand might also help with the maintenance of specific growth rate below the inhibitory threshold. Improved growth rates and recombinant protein productions can be achieved by alleviating detrimental effects of acetate by the addition of certain amino acids (e.g. Gly and Met) (Lee, 1996).

1.4.5 Point of Induction

Maximum productivity in HCDC of recombinant *E. coli* can be achieved through separation of growth phase from production phase. In order to achieve the phase's separation, in fed-batch cultures, the induction of the bacterial population may be delayed until a reasonable cell density is reached. The inducible expression systems can be induced by different mechanisms including temperature shift, addition of chemical inducers or pH changes (Choi et al., 2006).

The most widely used inducible promoters include T7 or *lac*-based promoters (*tac*, *trc*, *lac*, *lacUV5-T7 hybrid*, etc.) that can be easily induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Choi et al., 2006). Since high concentration of IPTG can lead to the inhibition of cell growth and recombinant protein production (Choi et al., 2000), determination of an optimal inducer concentration (Choi et al., 2000) and point of induction (Jeong and Lee, 1999; Yim et al., 2001) is of significant importance for the achievement of high productivity in HCDC of *E. coli*. In a work carried out by Doig et al. (2001) on recombinant production of CHMO (cyclohexanonemonooxygenase) in *E. coli*

using the L-arabinose inducible vector, the moment for induction was optimal when the oxygen transfer was maximised and the dissolved oxygen was zero. It is not willing to induce the industrial-scale production of recombinant proteins by the addition of IPTG due to its hindering price when it is required in higher amounts (Chauhan et al., 2001). Several attempts have been made to overcome this problem by designing different constitutive or inducible expression systems (Chauhan et al., 2001). For the former one, there are recent reports on the recombinant production of human leptin (Jeong et al., 2004) and tumor necrosis factor-α (Poo et al., 2002) in *E. coli* by HCDC technique using a new strong constitutive promoter from the D-amino acid aminotransferase gene of *Geobacillustoebii*.

Although IPTG had been reported to have adverse effects on cellular metabolism even in the absence of a foreign gene (Miao and Kompala, 1992), no disruption in fermentation progression observed upon induction of a non-recombinant *E. coli* hence excluding any intrinsic toxic characteristics (Lewis et al., 2004). In addition, IPTG is not metabolised unlike lactose, the other potential inducer, hence making it possible to separate the growth phase from production phase.

1.4.6 Plasmid Instability

In recombinant expression systems, maintenance of a high plasmid stability of the cell population during the cultivation process is directly proportional to the productivity. Plasmid instability is ascribed to either plasmid loss or some form of structural changes in plasmid referred to as segregational and structural plasmid instabilities, respectively.

The latter was encountered upon early inductions (Lewis et al., 2004) and accompanied by synthesis of anomalous proteins. Segregational plasmid instability arises from the conflict exists between transcription and plasmid replication (Parker and DiBiasiot, 1987) and the lower growth rate of plasmid-carrying cells than plasmid-free cells (Zhang et al., 2003; Xu et al., 2006). Increased transcription hampered segregation of plasmid molecules between mother and daughter cells and injured plasmid DNA. On the other hand, the increased transcriptional activity overburdened the cell's DNA repair capability. Slower growth rates arise from the additional metabolic burden on plasmid-carrying cells imposed by plasmid-related activities including the maintenance and replication of DNA plasmid and the recombinant gene expression. This is intensified under actual induction conditions due to the synthesis of target protein. In attempts to diminish the plasmid segregational instability, molecular and bioprocess strategies have been employed solely or in combination. As a molecular strategy, cells can be cultivated in antibiotic-selective pressure media for the inhibition of proliferation of plasmid-free cells to balance the additional metabolic burden on plasmid-carrying cell. Xu et al. (2006) has shown the positive effect of antibiotic on retention of plasmid stability with a more pronounced effect under the induction conditions. In a work by (Gupta et al., 1995), retention of plasmid stability was examined as a function of temperature and within the temperature range of 25 to 42°C, temperatures below 33°C damaged the plasmid stability. Lower plasmid stability at reduced cultivation temperatures was attributed to: the reduction in plasmid copy number (Oscam et al., 1992); triggering of plasmid rejection instead of reduced growth rate (Gupta et al., 1995) since synchronization between plasmid replication rate and cell division rate at lower growth

rates led to improved plasmid stability in minimal media (Gupta et al., 1995; Tomazetto et al., 2007).

A combination of different strategies can be used to improve the final achievable cell concentration (Lee, 1996).

1.5 Aims and Objectives

The purpose of the present study is to develop bioprocess-based strategies for improvement of volumetric productivity; protein localisation in the periplasm — the originally intended target for subcellular location in this study — and periplasmic product retention throughout the process. Glycerol was preferred as the main carbon source because of its solubility and that it supports growth of cells at lower rates. Fed-batch fermentation was employed to maintain limiting concentrations of glycerol throughout the process. Temperature of the operation was studied as the factor influencing cell growth and protein folding and stability. Low temperature for the induction phase was selected with the purpose to synchronise the cell division rate to the plasmid replication rate to improve the plasmid stability and subsequently the productivity in combination with antibiotic addition to attenuate growth of plasmid-free cells. In addition, it aimed to tune the synthesis rate of target protein to the capacity of secretory machinery. Effect of lower temperature of growth was also investigated. As in form of preliminary studies, shake-flasks were performed to find out the severity of growth cessation and recombinant system productivity upon early inductions.

2. CHAPTER 2 – MATERIALS AND METHODS

2.1 Materials

2.1.1 Organism

The tetracycline resistant *E. coli* CLD048 harbouring the secretion vector plasmid pAVE046, which contains the gene coded for antibody fragment Fab D1.3, was supplied by Avecia Biotechnology (Birmingham, UK). Expression of Fab D1.3 takes place through secretion of light and heavy chains into the periplasm to facilitate the formation of an interchain disulphide bond to create antibody fragment.

2.1.2 Chemicals

Table 2.1 Chemicals List

Chemical	Supplier
Yeast Extract	Becton, Dickinson and Company, MD, USA
Tryptone	Becton, Dickinson and company, MD, USA
Ammonium hydroxide	Fisher Scientific, Leicestershire, UK
Magnesium sulphate heptahydrate	Fisher Scientific, Leicestershire, UK
Phosphoric acid	Fisher Scientific, Leicestershire, UK
Calcium chloride dehydrate	Fisher Scientific, Leicestershire, UK
Manganese sulphate monohydrate	Fisher Scientific, Leicestershire, UK
HPLC grade methanol	Fisher Scientific, Leicestershire, UK
Nutrient agar	Oxoid, Hampshire, UK
Phosphate buffered saline (PBS)	Oxoid, Hampshire, UK
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Thermo Scientific, IL, USA
Ammonium sulphate	Sigma-Aldrich Co, MO, USA
Glycerol	Sigma-Aldrich Co, MO, USA
Potassium phosphate monobasic	Sigma-Aldrich Co, MO, USA
Potassium phosphate dibasic	Sigma-Aldrich Co, MO, USA
Citric acid	Sigma-Aldrich Co, MO, USA
Tetracycline	Sigma-Aldrich Co, MO, USA
Ferrous sulphate heptahydrate	Sigma-Aldrich Co, MO, USA
Zinc sulphate heptahydrate	Sigma-Aldrich Co, MO, USA
Sodium molybdate dehydrate	Sigma-Aldrich Co, MO, USA
Copper (II) sulphate pentahydrate	Sigma-Aldrich Co, MO, USA
Boric acid	Sigma-Aldrich Co, MO, USA
Trizma-Base	Sigma-Aldrich Co, MO, USA
Tetrasodium ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich Co, MO, USA
Sucrose	Sigma-Aldrich Co, MO, USA
Sodium carbonate	Sigma-Aldrich Co, MO, USA
Sodium hydrogen carbonate	Sigma-Aldrich Co, MO, USA
Chicken egg white lysozyme (HEWL)	Sigma-Aldrich Co, MO, USA
Albumin from bovine serum (BSA)	Sigma-Aldrich Co, MO, USA
Tween 20	Sigma-Aldrich Co, MO, USA
Goat anti-human Fab peroxide conjugate	Sigma-Aldrich Co, MO, USA

2.1.3 Consumables

TMB 2-Component Microwell Peroxidase Kit was purchased from Kirkegaard & Perry Laboratories (MD, USA).

2.1.4 Equipments

Table 2.2 Equipments List

Equipment	Manufacturer
Shaker Incubator	Gallenkamp, Germany
FerMac 310/60 Fermenter System	Electrolab, Gloucestershire, UK
CE7500 Double Beam, UV/VIS Aquarius Spectrophotometer	Cecil Instruments Ltd, Cambridge, UK
Gallenkamp Hotbox Oven	Riley Industries Ltd, Aldridge, UK
CC-1 Colony Counter	Progen Scientific Ltd, London, UK
5415D Microcentrifuge	Eppendorf, Hamburg, Germany
AK120 Microplate Incubator Shaker	Infors HT, Bottmingen, Switzerland
Promega GloMax-Multi Microplate Multimode Reader	Turner BioSystems Inc., CA, USA

2.2 Experimental Methods

2.2.1 Inoculum Preparation

Starter cultures were prepared from Luria Bertani (LB) medium, consisting of 1% NaCl, 1% tryptone, and 0.5% yeast extract, in 500 mL baffled conical shake flasks to the working volume of 120 mL and its pH was adjusted as 7 using ammonium hydroxide (NH_4OH). Post-sterilization addition of tetracycline antibiotic was made to the culture medium after it had cooled to the running temperature to yield a final concentration of

15 mg.L^{-1} using a 0.22 μm sterile filter. Subsequently, the culture was inoculated by aseptic addition of 120 μL of the frozen bacterial stock to gain 0.1% inoculum. The starter culture was incubated at the temperature of 37°C for the temperature downshift fermentation and the shake-flask cultivations and at 30°C in case of the low constant temperature fermentation in an orbital shaker at the speed of 200 rpm over night.

2.2.2 Fed-Batch Fermentation

Basal fermentation medium was made up according to Table 2.3.

Table 2.3 Medium Composition – Shaded portion indicates the post-sterilisation additions

Component	Concentration
$(NH_4)_2SO_4$	14 g.L^{-1}
Glycerol	35 g.L^{-1}
Yeast Extract	20 g.L^{-1}
KH_2PO_4	2 g.L^{-1}
K_2HPO_4	16.5 g.L^{-1}
Citric Acid	7.5 g.L^{-1}
1 M $MgSO_4.7H_2O$	10 mL.L^{-1}
1 M $CaCl_2.2H_2O$	2 mL.L^{-1}
Tetracycline (of a 15 mg.mL^{-1} stock)	1 mL.L^{-1}
Trace Metal Solution (according to Table 2.4)	34 mL.L^{-1}
Antifoam AF204 (polypropylene glycol 2,000)	0.2 mL.L^{-1}

Table 2.4 Composition of Trace Metal Solution

Component	Concentration
$FeSO_4.7H_2O$	3.36 g.L^{-1}
$ZnSO_4.7H_2O$	0.84 g.L^{-1}
$MnSO_4.H_2O$	0.51 g.L^{-1}
$Na_2MoO_4.2H_2O$	0.25 g.L^{-1}
$CuSO_4.5H_2O$	0.12 g.L^{-1}
H_3BO_3	0.36 g.L^{-1}
H_3PO_4	48 mL.L^{-1}

Again ammonium hydroxide was used to adjust pH of 7 for the medium. Autoclave-sterilized fermentation vessel containing basal medium allowed cooling to the operation temperature. Fermentation was started by inoculation with the 120 mL starter culture (described in section 3.2.1) and was performed in fed-batch mode (4% inoculum to medium ratio). Fab antibody expression was induced by aseptic addition of filter-sterilized isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.15 mM at the point that optical density (OD_{600nm}) reached 50 ± 5. The fermenter was equipped with pH probe, dissolved oxygen (DO) probe, and temperature sensor, to measure pH, DO, and temperature online respectively. pH was assured to maintain above 7 by automatic feedback control and an internal port for NH_4OH solution. DO value was dependent on agitation rate. Two sets of fermentations were designed to study the effect of temperature on expression process: one with a temperature downshift from 37 to 30°C at the point of induction; the other with running the whole fermentation process at a lower temperature of 30°C. In case of the temperature downshift run, while the optical density was about to gain the desired value for induction, temperature was switched using a cooler to overcome the metabolic heat generated by *E. coli* cells. Cell growth was monitored by taking samples hourly and measuring the optical density by spectrophotometer. The initial working volume of the fed-batch process was operated in a batch mode until glycerol was exhausted. While a depletion in glycerol was observed by an increase in DO, the fermenter was fed with the glycerol/magnesium sulphate feed (714 $g.L^{-1}$ glycerol; 30 $mL.L^{-1}$ of 1 M $MgSO_4.7H_2O$) at a constant rate of 11.0 ± 2.0 $g(glycerol).L^{-1}.h^{-1}$ (45 $mL.h^{-1}$). Samples were taken each hour for single unit measurement (colony-forming $unit.mL^{-1}$ or $CFU.mL^{-1}$) as well as for osmotic-shock analysis during the post-induction phase. The fed-batch fermentations

were carried out in laboratory-scale in a 5 L cylindrical glass bioreactor of 162 mm diameter and 300 mm total height with a working volume of 3 L. The vessel was fitted with two identical 82 mm six bladed radial flow paddle type impellers and a distance of 80 mm was considered from the lower impeller to the bottom of the vessel and to the upper impeller. Three equally spaced stainless steel baffles with 15 mm width were also fitted within the bioreactor to aid mixing. Air was sterilized by passage through a steam sterilisable 0.2 μm PTFE filter and was then sparged at the rate of 1 vvm (litre gas per litre broth per minute) into the bottom of the reactor through a L-shaped air sparger. The head plate of bioreactor contained ports for the pH probe, DO probe, and temperature probe and addition ports for antifoam, inoculum, base, and feed additions and for sample extraction. An electrical heating jacket and a cooling valve connected to a cooling finger were used to maintain the desired temperature in reactor. The feed was pumped to the reactor at the required rate through a peristaltic pump for fed-batch fermentation.

2.2.3 Shake-Flask Cultivation

A set of shake-flask cultivations were performed to investigate how the point of induction influences the expression of D1.3 Fab antibodies. It involved 3 shake flask cultivations, one without induction and the other two induced at OD_{600nm} of 0.5 and 1 with IPTG. Each experiment was performed in duplicates to reduce experimental and random errors and to improve the precision of results. In case of induced cultivations, samples were taken each hour before induction to monitor cell growth by measuring the OD_{600nm}. During the non-induced cultivation and the post-induction phase of induced

cultivations, shake-flasks were sampled at 2, 4, 6, and 24 hrs post-induction for OD_{600nm} and CFU measurements and for ELISA analysis. Fermentation basal medium and post-sterilization additions were all made up according to section 2.2.2 but for an initial working volume of 100 mL. The pH of the medium was adjusted to 7 using ammonium hydroxide prior to incubation. Cultivations were all carried out in baffled conical shake-flasks of 500 mL volume at 37°C and 200 rpm in an orbital shaker. All shake-flasks were inoculated with 1% inoculums (section 2.2.1) and induced with 0.15 mM IPTG at the designated point of induction where proposed.

2.3 Methods of Analysis

Monitoring and controlling of the bioprocess is carried out throughout the two modes of on-line and off-line/at-line analyses to maintain the desirable conditions for the production of target product and to ensure achievement of the optimal biomass and product yield. On-line is the analysis directly from bioreactor and is performed in two forms of *in-situ* or *ex-situ*. *In-situ* measurement is taken place in direct contact with the fermentation broth under the processing conditions such as measurement of DO, pH, and temperature. *Ex-situ* analysis is carried out away from the fermenter via a fitted window or through a flow from system. Measuring the analytes and the biomass which monitors the progress of the cultivation is performed conventionally through off-line analysis, where the sample is taken away from the bioreactor. Analytes that need to be monitored include the carbon source (like glucose) or the nitrogen source (like ammonium) to avoid accumulation of detrimental concentrations of unwanted organic by-products like acetate or formate or subsequent oxygen-deficiency. Since off-line

analysis is time-consuming and labour-intensive, several attempts have been made to design simpler and closer to real time alternatives which has led to the development of *in-situ* measurements via a flow stream system (Kousar, 2008). However, there are several factors limiting the application of such methods like specificity to one-analyte, sensitivity to environmental conditions or necessity of frequent recalibrations.

2.3.1 Traditional Microbiological Analysis

Monitoring the cell proliferation and viability is essential throughout any microbial processes. Since the cell number and individual cell physiological state determines the process performance, accurate measurement of biomass concentration is of real concern for process control (Hewitt and Nebe-Von-Caron, 2001). Accurate measurements facilitate determination of an optimum concentration for harvesting the products and an appropriate induction point for inducible systems. This helps towards the achievement of high product yields and detection of detrimental effects of dead and dormant cells on synthesis of target products (Hewitt and Nebe-Von-Caron, 2001; Kousar, 2008).

2.3.1.1 Optical Density

Bulk measurement is most often used as a simple and quick way to measure biomass proliferation and gives a good estimate of cell growth, even though it provides no information on physiological state, composition, and size of cultured cells (Hewitt and Nebe-Von-Caron, 2001; Pope, 2006). Studying the results from bulk analyses is based on the assumption that the population is in homogeneous state (Hewitt and Nebe-Von-

Caron, 2001; Pope, 2006). Correlating the optical density to dry cell weight is a way to identify if any changes occurred in size and shape of the cells during cultivation. The non-linear correlations observed throughout the work by Pope (2006) on characterization of therapeutic grade plasmid produced by recombinant *E. coli* is an insight to the fact that optical density is not solely reliable for measuring the biomass concentration. Amount of light detected by spectrophotometer detector is inversely proportional to the bacterial population or in other words to the observed turbidity. It shows the influence of number, size, and morphology of cells on the absorbance (optical density) of the sample (Pope, 2006). Cell growth was monitored by measuring the optical density at a wavelength of 600 nm (OD_{600nm}) using a double-beam spectrophotometer. Samples might require dilution with PBS before analysis as readouts were only taken within the range of 0-0.7 absorbance units.

2.3.1.2 Colony-Forming Units

The number of colony-forming units ($CFU.mL^{-1}$) was measured by making serial dilutions of the samples taken at certain time intervals from the bioreactor. 1 mL of sample was diluted in 9 mL of PBS in each test tube, mixed well, and then 100 µL was spread on the agar plate previously prepared for the cultivation of bacteria to measure the propagation and culturability of the bacterial population. The plates were incubated over night at the respective operating temperature and then the numbers of colonies were counted by a colony counter. Plasmid-specific agar plates were also prepared by the addition of plasmid-specific antibiotic, tetracycline, to the agar solution to give a figure on plasmid-carrying cells.

2.3.2 Osmotic Shock Treatment

Table 2.5 Composition of Osmotic Shock Solutions

Solution	Composition
Osmotic Shock Solution 1 (OS solution 1)	Trizma-Base 2.64 $g.L^{-1}$
	Tris-HCl 0.39 $g.L^{-1}$
	Tetrasodium EDTA dihydrate 1.04 $g.L^{-1}$
	Sucrose 200 $g.L^{-1}$
	pH 8 at 5°C
Osmotic Shock Solution (OS solution 2)	Trizma-Base 2.64 $g.L^{-1}$
	Tris-HCl 0.39 $g.L^{-1}$
	Tetrasodium EDTA dihydrate 1.04 $g.L^{-1}$
	pH 8 at 5°C

Osmotic shock (OS) treatment was used in this study as a cell disruption method to fractionate the total Fab D1.3 produced based on subcellular location. The 1 mL sample taken through the course of fermentation for this purpose was centrifuged in a microcentrifuge at 16,100 g for a couple of minutes. The supernatant was decanted and stored at -20°C which represents the culture broth. The cell pellets were re-suspended by vortexing in 1 mL of OS solution 1 and then the resuspended cells were allowed to incubate on ice for 10 minutes. After spinning down, the supernatant was decanted and stored for the measurement of antibody fragment concentration within the periplasm. The same procedure was performed this time using OS solution 2 to reveal the cytoplasmic content.

2.3.3 ELISA

In chemical assays, a specific chemical reaction occurs that produces a colour change and the intensity of the colour is measured with a spectrophotometer to achieve the absorbance. To calculate the unknown concentrations, a correlation between absorbance and concentration needs to be developed from a calibration curve using the data on standards. High temperatures are often required for incubation and to indicate the chemical change, toxic chemicals are usually necessary. Component specific assay kits work on a similar principle, however, an enzyme is used instead to react with the component of interest. The difficulties associated with the assay kits are that they are tedious, time-consuming, and component specific, they need sample pre-treatment, the data are operator-dependent and that the sample is consumed (Kousar, 2008).

Table 2.6 Composition of ELISA Solutions

Solution	Composition
Coating buffer	Na_2CO_3 1.59 $g.L^{-1}$
	$NaHCO_3$ 2.93 $g.L^{-1}$
	pH 9.6
Coating with Lysozyme	Coating buffer
	Lysozyme 1 $g.L^{-1}$
Blocking buffer	10 PBS tablet
	BSA 10 $g.L^{-1}$
Washing buffer	10 PBS tablets
	Tween-20 1 $mL.L^{-1}$
Detection antibody	Blocking buffer 20 mL
	Goat anti-human Fab peroxidase conjugate 2 μL

Fab concentration in each cell compartment was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) in replica following osmotic shock treatment. 96-well

ELISA plate was loaded with 100 μL of coating buffer per well and incubated at 4°C overnight. After discarding the coating buffer, the ELISA plate was blocked with 200 μL of blocking buffer per well and incubated at 37°C for 1 hour. A dilution series was then created on a polypropylene round bottomed 96-well plate with 180 μL of neat samples in each well of one row and 120 μL of blocking buffer in the remaining wells. Serial dilutions were carried out by transferring 60 μL from top well to next well down, mixing well, and repeating down the plate. After washing the ELISA plate with washing buffer and tap drying for three times (300 μL per well), 100 μL of serial dilutions from each well of the dilution plate was transferred to the exact well of the ELISA plate and then the ELISA plate was incubated on a shaker at 37°C for an hour. The washing and tap drying step was performed. Afterwards for antibody detection, each well was loaded with 100 μL of detection antibody in blocking buffer and subsequent incubation for 1 hr at 37°C was required. After washing and tap drying, 100 μl of peroxidase substrate was added to each well and the plate was allowed to incubate for 10 minutes at 25°C. The reaction was stopped with 100 μL of 1 M H_3PO_4 per well and absorbance reading was undertaken at 450 nm using the plate reader. To calculate the concentration of each sample based on the measured absorbance, a correlation needs to be made between the absorbance and the known concentration of the recombinant antibody fragment.

3. CHAPTER 3 – RESULTS AND DISCUSSIONS

With the aim to improve the plasmid-stability and consequently the *E. coli* CLD048 recombinant system productivity, a two-stage cultivation strategy was applied for fed-batch fermentations. That was by integration of molecular- and process- based strategies with antibiotic addition in favour of plasmid-bearing cells and induction at high enough cell densities for separating the growth phase from the production phase. The fed-batch fermentations were performed in two categories, one with conducting the whole cultivation process at a sub-optimal temperature for bacterial growth and the other with growing the cells at a temperature optimal for bacterial growth followed by lowering the temperature for the production phase to comparatively assess the behaviour of cells towards volumetric productivity and subcellular distribution. In order to investigate the chance of minimizing detrimental effects of induction by inducing early through logarithmic phase, preliminary studies on point of induction were performed in the form of shake-flask fermentations.

3.1 Influence of operational conditions on culturability and productivity of the recombinant *E. coli* CLD048

The process that is central to this thesis entails fed-batch fermentation of the bacteria in a laboratory-scale stirred-tank reactor, followed by induction with IPTG for expression of recombinant anti-lysozyme antibody fragment.

In recombinant expression systems, maintenance of a high plasmid stability of the cell population during the cultivation process is directly proportional to the productivity (Gupta et al., 1995; Zhang et al., 2003). In attempts to diminish the plasmid segregational instability, molecular and bioprocess strategies have been employed solely or in combination. As a molecular strategy, cell cultivation in an antibiotic-selective pressure media modulates post-segregational effects by killing or inhibiting proliferation of the plasmid-free cells (Zhang et al., 2003). We employed a combination of the two types of strategies; a two-stage cultivation entailing a growth phase in favour of plasmid-carrying cells by addition of antibiotic followed by the production phase induced by IPTG at high enough concentration of biomass. To separate the growth phase from the production phase, $OD_{600nm} \sim 50 \pm 5$ was designated as the point of induction.

A survey within the literature revealed that conducting the fermentation at a lower temperature brings about the advantages of synthesis rate of target protein being tuned to the translocation capacity of secretory pathway (Balagurunathan and Jayara, 2008; Dragosits et al., 2009) and the growth rate being reduced. The resultant reduction in growth rate lowers the chance of acetic acid formation (Sandén et al., 2003) and leads to synchronization between plasmid replication rate and cell division rate under the conditions that the temperature is set well above the threshold triggering plasmid rejection (Gupta et al., 1995). Fed-batch fermentation was conducted with growing *E. coli* cells at the sub-optimal temperature of 30°C. In a comparative approach, a strategy of temperature downshift at the point of induction was employed to investigate the influence on the volumetric productivity and the subcellular distribution of the recombinant product. To promote the cell growth, *E. coli* cells were cultivated at 37°C in

growth phase to reach a population designated for induction, followed by a shift to a lower temperature of 30°C for the production phase in favour of plasmid-bearing cells and to aid correct folding.

Figure 3.1 illustrates the parameters that were monitored online throughout the two fermentation progresses (dissolved oxygen (DO) and pH) along with the optical density. Agitation speed controlled DO above 30% air-saturation by variations within a range of 250-1000 rpm. The air flow rate was set at 1 vvm. Ultimately, oxygen uptake rate exceeded the oxygen transfer rate as indicated by continuous fall in DO while agitation was operated at the upper limit rate. Afterwards, the level of DO started to increase reflecting the carbon source depletion. Subsequently, feeding of glycerol stock solution was started at the pre-determined rate by manual activation of a pre-calibrated pump. Since there was no reliable on-line measurement of glycerol concentration, DO was used as a surrogate. pH was controlled well throughout the process but a dramatic drop to a value below the lower limit of the set-point range (6.8-7.2) was observed just before feeding was started. This can be a sign of organic acids formation under oxygen-limiting conditions which was adjusted by automatic addition of ammonium hydroxide. Cells were harvested upon a dramatic rise in DO and an onset of increase in pH concomitant with a progressive drop in OD which show the switch to unhealthy conditions for cell cultivation. The rise in pH might be due to the accumulation of ammonium ions excreted by cells following carbon exhaustion.

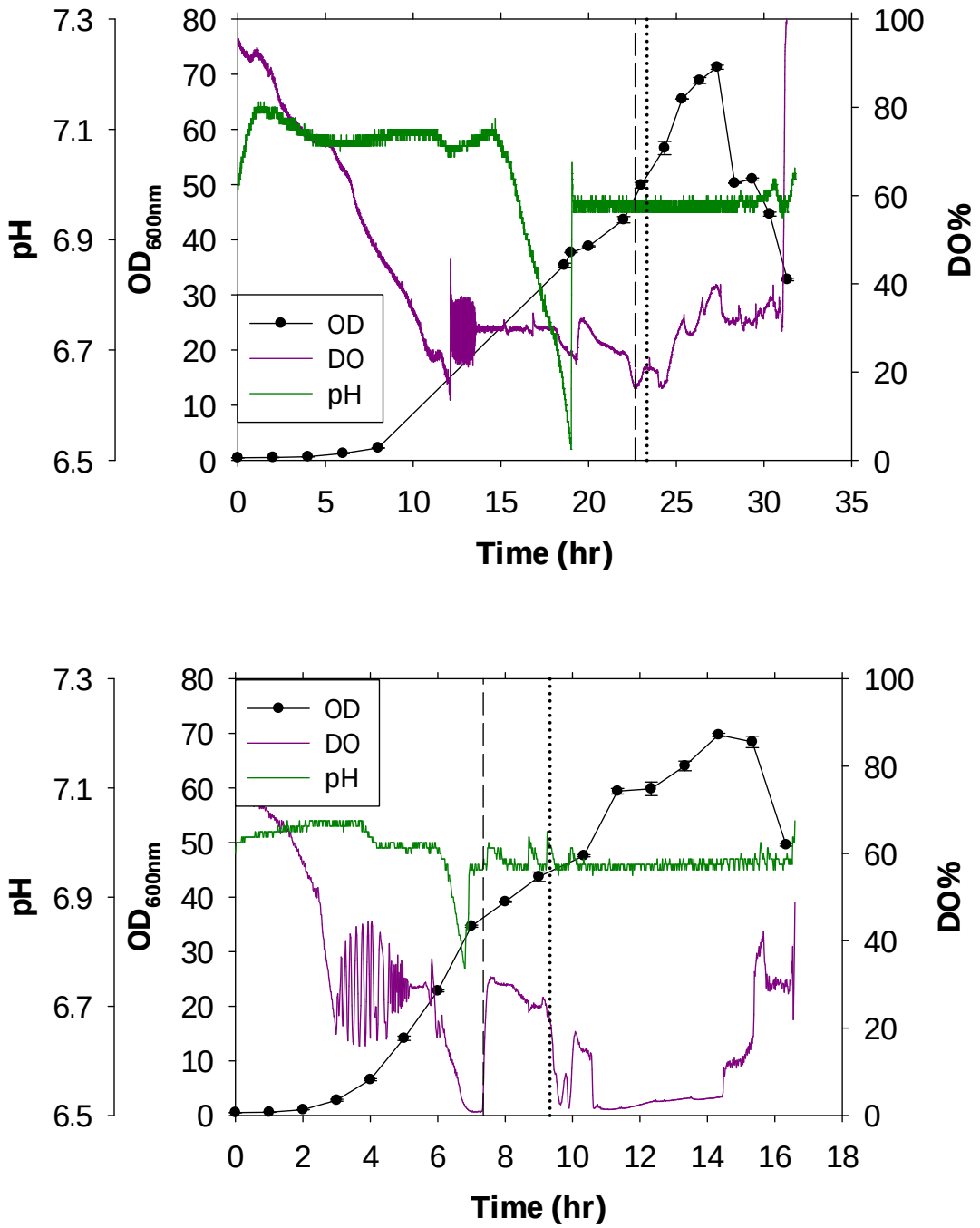

Figure 3.1 Process parameters monitored throughout the course of fed-batch fermentations of recombinant *E. coli* CLD048. Top: the whole cultivation process was performed at a constant temperature of 30°C; Bottom: the induction was carried out at a temperature (30°C) lower than that of the growth phase (37°C). Dashed line shows the feeding point and the dotted line is where 0.15 mM IPTG was added. All points plotted for OD_{600nm} are mean values from replicate analyses and the error bars show the standard deviation of the data from which the mean was calculated.

Two more analyses were performed in case of low temperature cultivation; the concentration of residual glycerol in the medium (by means of HPLC analysis) and number of plasmid-carrying cells in terms of CFU.mL^{-1} (see Figure 3.2). The number of viable cells (CFU.mL^{-1}) and viable cells carrying plasmid (CFU.mL^{-1}) follow quite similar path throughout the whole process. Fluctuations in plasmid stability may be explained in terms of variations in the concentration of dissolved oxygen. Higher oxygen mass transfer rate ended in lower plasmid stability (Tomazetto et al., 2007) and Gupta et al. (1995) showed the need for optimization of oxygenation to minimise the plasmid instability. It has been reported that the plasmid stability is independent of pH over a range of 5-8 (Gupta et al., 1995). After 2 hours of cultivation, the concentration of glycerol in medium reached to one seventh of its initial concentration (35 g.L^{-1}). The next sample taken for glycerol measurement was after 20 hrs of cultivation proving that the medium was getting depleted in carbon source as reflected in a rise in DO. After addition of feeding solution, glycerol concentration slightly increased but 2 hrs later there was zero accumulation of glycerol highlighting the close match between the uptake rate and the transfer rate of glycerol and the maintenance of limiting concentrations of carbon source as aimed by the application of fed-batch strategy.

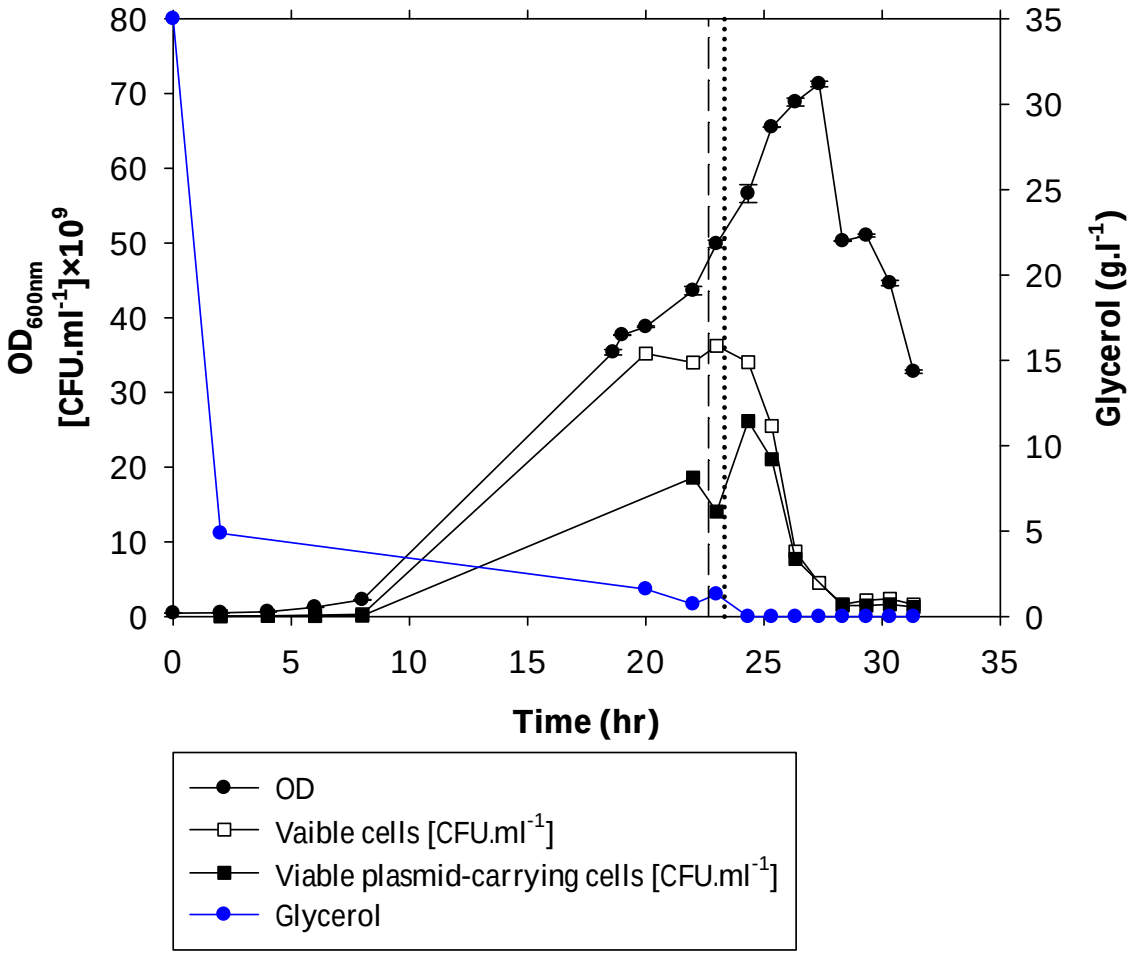

Figure 3.2 Further analyses which performed for the cultivation at the lower temperature of 30°C. Dashed line shows the feeding point and the dotted line is where 0.15 mM IPTG was added. All points plotted for OD_{600nm} are mean values from replicate analyses and the error bars show the standard deviation.

Figure 3.3 shows the optical density, $CFU.mL^{-1}$, and product concentration for the two fermentation runs. Osmotic shock solutions were used for cellular fractionation to reveal the content of Fab in each compartment; supernatant shows the extracellular milieu content and the osmotic shock solutions 1 and 2 used to determine the antibody concentration in periplasm and cytoplasm respectively. Following osmotic shock, ELISA was used as the quantification method to determine the Fab concentration in each

compartment. Total Fab produced was simply defined as the summation of Fab contents in three compartments. Fab antibody was expected to be expressed through secretion of light and heavy chains into the periplasm for the formation of an interchain disulphide bond.

By cultivation at the lower temperature (30°C), a longer lag phase and a slower growth ended in the achievement of the maximum biomass, in terms of optical density, similar to that of the temperature downshift operation (OD_{600nm} ~ 70) but 14 hrs later through the fermentation process. In terms of $CFU.mL^{-1}$, both fermentations reached the maximum right after the induction and entered the decline phase while broth turbidity was still increasing throughout the production phase implying the entrance to the viable but nonculturable (VBNC) state. Appearance of VBNC cells has long been documented for more than 70 bacterial species under adverse environmental conditions as in starved *E. coli* cells (Pinto et al., 2011). Even though these cells are not detectable by routine microbiological culturing methods (Nilsson et al., 1991; Oliver, 2010), their detectable metabolic functions revealed diminution in nutrient transport, respiration rates, and macromolecular synthesis but gene expression has been demonstrated (Arana et al. 2007).

Given the increase in optical densitometry being the corollary of increase in bacterial cell numbers, one might expect the similarity in culture turbidity between the two fermentations to extend to the CFU analysis. $CFU.mL^{-1}$ attained for the temperature downshift operation was two-fold greater than that of the constant temperature fermentation which can probably be due to the temperature difference leading to

different cell size and mass. Changes in cell length are regarded as phenotypic responses to growth conditions, some conditions favouring and others inhibiting division amongst short organisms (Maclean and Munson, 1961). Other than at extremes, temperature seems to have insignificant effect on the cell size (Shehata and Marr, 1975). The cell volume distributions for cultures of *E. coli* ML30G growing in steady state in a minimal medium at temperatures in the range of 15 to 30°C were indistinguishable but at 35°C the distribution was slightly shifted towards smaller volumes (Shehata and Marr, 1975). Other than the differences in cell size and mass, the probable reason behind the disparate results obtained in the present study could be that low temperature cultivation heightens the appearance of VBNC.

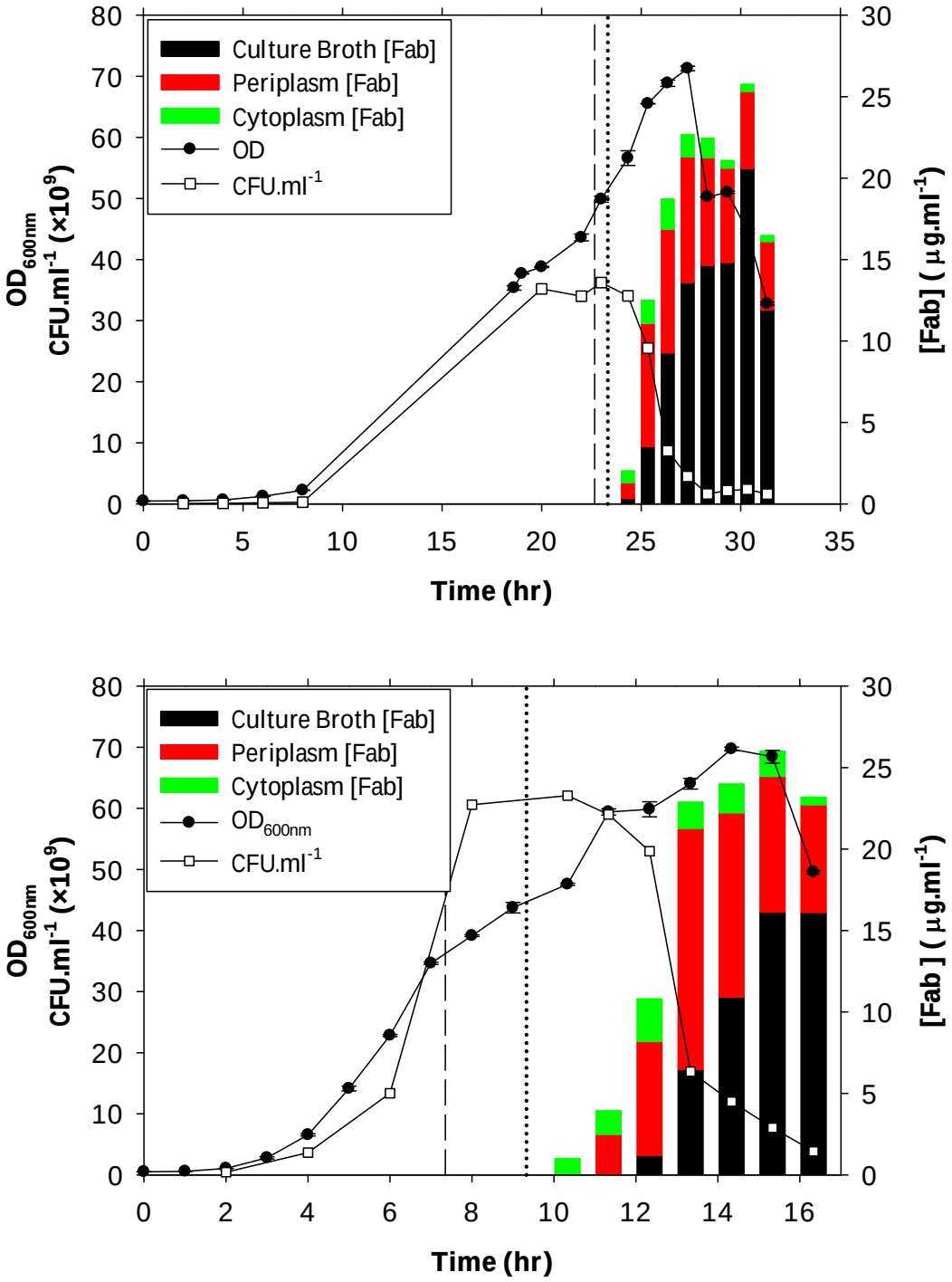

Figure 3.3 Product concentrations in cellular compartments, broth turbidity, and cell numbers for fed-batch fermentations of recombinant *E. coli* CLD048. Top: low temperature fermentation; Bottom: temperature downshift fermentation. Dashed line is where the feeding was started and the dotted line shows where 0.15 mM IPTG was added as an inducing agent. All points plotted for OD_{600nm} are mean values from replicate analyses and the error bars show the standard deviation.

Cell cultivation under low temperature conditions lengthened the production phase, i.e. from the moment of IPTG addition, by one hr compared to the temperature downshift operation (Figure 3.3). In terms of total Fab content as quantified by ELISA, both fermentations reached a maximum of about 26 $\mu g.mL^{-1}$ one hr before the termination of fermentation with the majority found in the culture broth; 80% and 62% for low constant temperature cultivation and temperature downshift strategy, respectively. Temperature downshift at point of induction resulted in reduced levels of basal expression of Fab antibody as compared with the low temperature cultivation. Only after 2 hrs from induction, antibody content in cells cultivated under low temperature conditions reached half of the maximum value, whereas this value was about 15% for temperature downshift strategy. Four hrs through the production phase, the two cultures reached similar concentrations of antibody (~ 23 $\mu g.mL^{-1}$; about 70% of the maximum concentration). In case of the temperature downshift strategy, the antibody concentration gradually increased afterwards to reach the maximum value at 6 hrs post-induction and one hr before the fermentation termination. For cultivation at low temperature, after achievement of 70% of maximum concentration at 4 hrs post-induction, slight decrease was observed over the next 2 hrs as the culture entered death phase during which the concentration in cytoplasmic and periplasmic space decreased and the extracellular concentration slightly increased. Then the antibody concentration increased to reach its maximum one hr before the fermentation ended.

The fact that temperature downshift at point of induction decreased the basal expression of Fab antibody corroborates the observation by Zhang et al. (2003) that the induction at a temperature lower than that of the growth phase delayed the basal

expression of GLP-2 in *E. coli* via a similar mechanism to that of catabolite repression caused by glucose. Zhang et al. (2003) examined different culture conditions with the aim to attenuate the activity or the amount of T7 RNA polymerase since the RNA polymerase is so active that the basal expression of toxic target protein and the high level of transcription of target gene (even if innocuous) would highly diminish the plasmid stability. Antibiotic addition after induction, inducing at a lower temperature, and addition of glucose to the medium were studied and the last two conditions were found to be more effective in terms of plasmid stability and protein yield.

During the last hr of fermentation, target protein produced by cell cultivation at low temperature declined steeply (down from 25.73 to 16.45 $\mu g.mL^{-1}$) whereas slight diminution observed in case of temperature downshift strategy (from 26 to 23.18 $\mu g.mL^{-1}$). That was concomitant with the onset of death phase for temperature downshift strategy while low temperature cultivation entered the death phase of growth earlier through the production phase. This implies that application of temperature downshift strategy for induction lowers the chance of proteolytic degradation of expressed product, offering higher stability of target product throughout the production phase, which is quite important from down-stream point of view. The increase in product concentration that observed during the death phase of low temperature cultivation could be explained in terms of the IPTG that released to the medium from lysed cells along with the intracellular contents being taken up by viable cells.

While, cultivation at lower temperature reduced the cytoplasmic accumulation of target protein by 4-fold, periplasmic retention was improved by almost 18% by employing the

temperature downshift strategy. Increased leakage of recombinant Fab into the extracellular milieu of *E. coli* culture at lower cultivation temperatures has already been encountered in a previous study (Dragosits et al., 2011). Protein is released to the extracellular milieu either by release of cellular contents upon cell death/lysis or by leakage which is the selective passage of proteins through the outer membrane at which the cell remains functional recombinant protein-producing. Based on the data available, it can be concluded that the protein release to the extracellular milieu was caused by protein leakage instead of cell lysis since it commenced while OD was still increasing. As mentioned earlier, it is not safe to rely solely on OD since continuous increase in OD has been reported elsewhere at constant biomass concentration (DCW) (Vidal et al., 2003; Lewis et al., 2004). The progressive increase in OD might be arisen from the changes in cellular composition mainly caused by accumulation of expressed proteins instead of cell propagation. Hence, there is a possibility that the onset of cell lysis would be misjudged. To be more accurate, measurements of total cell protein is required as a method of quantifying cell lysis. The cell's ability to retain periplasmic proteins is a function of the structural integrity of the outer membrane which is determined by its compositions of lipids, proteins, and lipopolysaccharides (Bäcklunda et al., 2008). The composition changes occur upon genotypic and environmental changes. In the present study, the two strategies only differed in the temperature of the growth phase and they resulted in similar final product titres. However, since ELISA is only able to detect functional antibody fragments with intact complimentary determining region, able to bind to their antigen, the improperly folded or truncated proteins would not be determined and the total proteins produced would be underestimated. Therefore, more evidence is required to conclude whether the increased leakage by low temperature

cultivation is caused by the physiological-changes triggered by low temperature enhancing product leakage or simply by the increased amount of protein. For example, a more generic method for determination of protein concentration can be used like SDS-PAGE Gel analysis. In this study insufficient amounts of Fab produced to be detectable by this method.

Both fermentations achieved similar OD and product concentration. Specific productivity is determined in terms of DCW and one might assume that the DCW would be equal for both fermentations since they reached similar OD. Since DCW was not measured for fed-batch fermentations and significant changes in the relation between OD and DCW upon induction have been reported for different recombinant *E. coli* cultures (Lewis et al., 2004; Vidal et al., 2003) and that the induction conditions differed between the two fermentations, it is not plausible to consider similar final biomass concentrations.

The common thought is that the improved protein production accomplished by reducing the fermentation temperature is resulted from reduced levels of proteolytic activity and improved cell viability (Balagurunathanand Jayara, 2008, Ruiz et al., 2009). Balagurunathan and Jayara (2008) reduced the cultivation temperature to tune the synthesis rate to the translocation capacity to reduce the accumulation of recombinant secretory streptokinase in inner membrane of *E. coli* and the corresponding up-regulation of an inner-membrane protease. In contrast, Dragostis et al. (2009) detected no reduction in the levels of proteases by growth at lower temperatures. Instead, reduced levels of protein folding stress were found to be responsible for the 3-fold

increased productivity in secretion of 3H6 Fab fragment in *Pichia pastoris* at lower cultivation temperatures. In a comparative host study with the aim to investigate the common features of the response to temperature reduction independent of growth-related effects in different host organisms, secretory expression of the antibody Fab fragment 3H6 in chemostat cultures of four different prokaryotic and eukaryotic microbial host systems were studied (Dragosits et al., 2011). Out of four, three host organisms including *E. coli* and *Pichia pastoris* showed improvements in terms of productivity and the authors suggest that the common physiological changes leading to reductions in protein-folding stress and maintenance energy is responsible for improved productivity at reduced temperatures. Cultivation at high temperatures magnifies the chance of denaturation and aggregation of native proteins and subsequently increases the demand for heat shock and stress proteins including chaperons necessary for refolding and degradation processes (Dragostis et al., 2009). Owing to the narrow span of thermal stability of native proteins, growth at reduced temperature is accompanied by down-regulation of proteins involved in protein secretion and folding. However, it offers higher secretion capacity as a positive side effect because of the higher stability of proteins and the consequent reduced demand for protein refolding and degradation at low temperatures.

3.2 Influence of time point of induction on culturability and productivity of the recombinant *E. coli* CLD048

The induction conditions need to be optimized in terms of both specific concentration (the amount of IPTG per gram of biomass) and volumetric concentration of IPTG. The

latter is a key parameter over inducer transport rate, specific production rate, and cell physiological state, and the former one is a key over the specific productivity of recombinant protein (Pinsach et al., 2008). Here the focus is on specific concentration of IPTG and a constant volumetric concentration of 0.15mM was taken throughout this study since it falls within the optimum range for periplasmic expression of recombinant Fab in *E. coli* (Shibui and Nagahari, 1992). To optimise the point at which the induction takes place, the availability of protein expression tools at time of induction and the severity of growth impairment upon induction needs to be taken into consideration. For those strains with drastic decrease in growth and viability upon induction, mid-log or stationary phase induction allows achievement of high cell density and leads to higher productivity. In a study on the effect of inclusion bodies formation on cell physiological state of the recombinant *E. coli* encoding a model fluorescent protein AP50 (Lewis et al., 2004), detrimental effects were more profound in case of later inductions and culturability was regained after all under induction with higher specific concentrations of IPTG (0.5 mM IPTG; point of induction: $OD_{550nm} \sim 0.1—15$). To study the influence of IPTG addition on growth of the recombinant strain of *E. coli* CLD048, preliminary studies were performed in the form of shake-flask fermentations. The points of induction were selected early through logarithmic phase to study whether early induction would offer any advantages over late induction and to check the ability of cells to synthesize Fab antibody during early logarithmic phase.

Shake flask cultures are the most basic tool to control and monitor the growth and behaviour of microorganisms and are widely used in a variety of biological research environments. To study the effect of induction point on biomass production and

formation of Fab antibody, two sets of shake flask cultivations were performed with antibody production being induced by 0.15 mM IPTG at OD_{600nm} of 0.5 and 1 in a set of experiments with the data shown in Figure 3.4. The other set was conducted with no induction to study the behaviour of *E. coli* in the absence of inducer towards culturability and productivity (see Figure 3.5). The highest amount of biomass was achieved in the absence of inducer at the end of cultivation (OD_{600nm} ~ 12) and the lowest value (OD_{600nm} ~ 1.5) was while Fab production was induced at OD_{600nm} of 0.5. The non-induced culture produced 0.75 $\mu g.ml^{-1}$ Fab by the end of cultivation which was comparable to those from induced cultures (about 1.1 $\mu g.ml^{-1}$) indicating the leakiness of the promoter allowing expression of the target protein-coding gene in the absence of inducer. Though the two induction points ended in similar product titres, they differed in basal expression of the antibody. With the later induction (induction at OD_{600nm} ~ 1), the product concentration reached its maximum at the first sampling point (2 hours after induction) and remained rather constant throughout 24 hours incubation as did the percentage within each compartment. While the cell culture was induced at OD_{600nm} of 0.5, half of the maximum was attained at the first sampling point and the concentration almost doubled at the next sampling point and reached a plateau while the periplasmic portion remained constant and the extracellular content increased. Cell lysis accounts for the release of target protein to the extracellular milieu as it was concomitant with a twofold decrease in broth turbidity and a fourfold decrease in cell number.

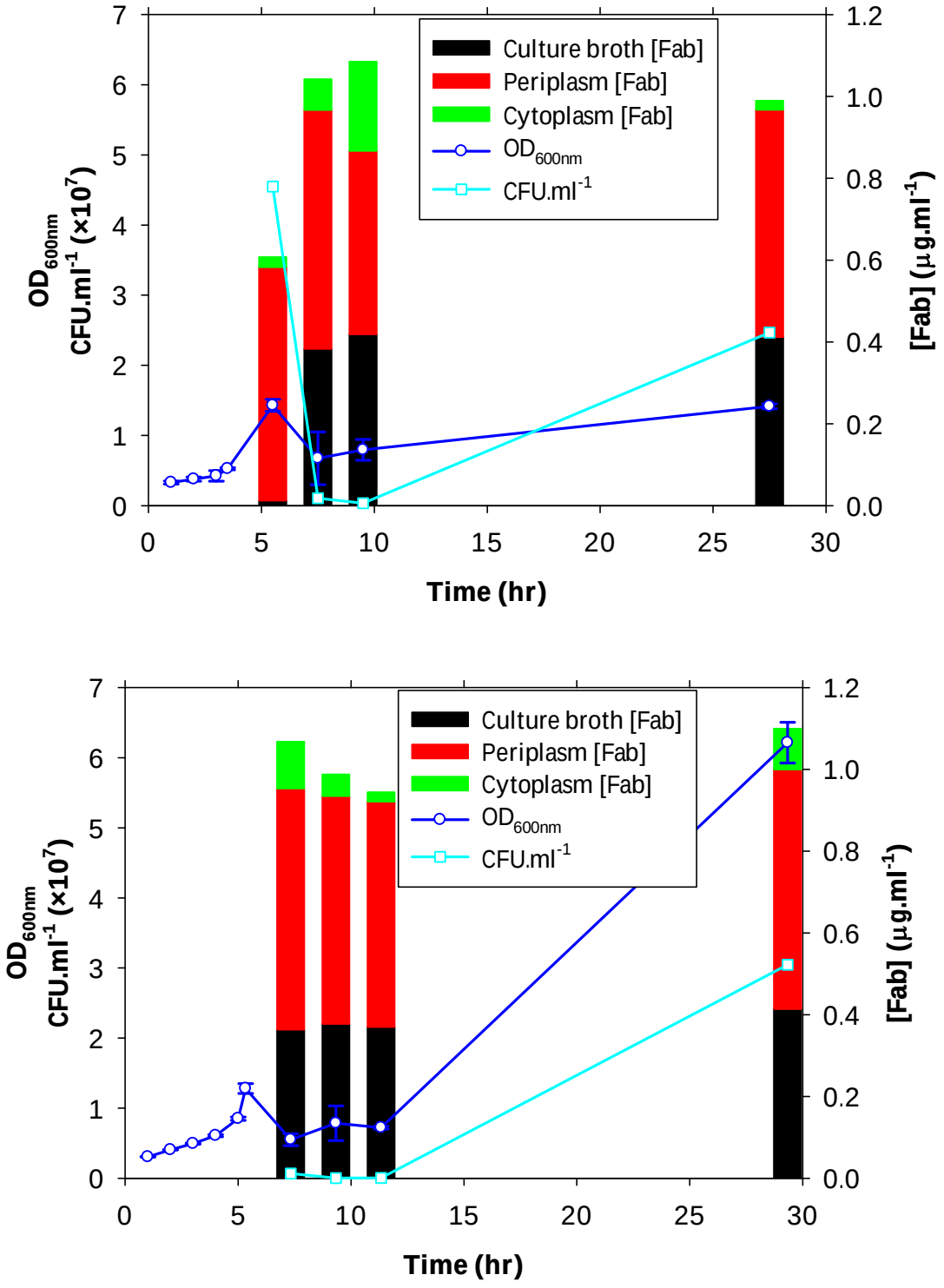

Figure 3.4 ***E. coli*** **CLD048 induced shake-flask fermentations. Top: the culture was induced at OD_{600nm} of 0.5; Bottom: induction took place at OD_{600nm} of 1. 0.15mM IPTG was used as the inducing agent. All points plotted are means values from replicates within each experiment. The error bars show the standard deviation of the data.**

Cultivation with no-induction was performed in replicates to monitor the growth characteristics of the recombinant strain. Want (2010) studied the same recombinant strain of *E. coli* CLD0048 in terms of physiological state during the course of fermentation using multi-parameter flow cytometry. It was reported that the cells from uninduced shake-flask cultivation entered the stationary phase in about 10 hours after reaching a maximum OD_{600nm} of 27, whereas in my experiments after about 25 hours of incubation the cells were still in logarithmic phase with the maximum OD_{600nm} of 12. The longer lag time and far lower specific growth rate could be explained in terms of the differences in inoculum size and operating volume and the fact that Want (2010) incubated a series of shake flasks enough for the time points of sampling eliminating any detrimental effects caused by the removal of flask from operative conditions for sampling. In this study, the percent inoculum and the operating volume were half the size and twice as high as those applied by Want (2010), respectively. A lower volume of liquid in a baffled flask of the same size improves agitation of the contents aiding mass transfer and provides a larger available surface area for oxygen transfer at air-liquid interface. Under optimum conditions, growth characteristic of a cell population is independent of inoculum history and is a sole function of environment (Robinson et al., 2001). Whereas cell populations suffering from starvation (Batchelor et al., 1997), heat injuries (Stephens et al., 1997), and inhibitory concentrations of NaCl (Robinson et al., 2001) exhibited longer and more variable lag time at small inoculum size. Effect of inoculum size under stressful conditions can be explained in terms of the requirement for chemical signalling (Batchelor et al., 1997), or some kind of medium conditioning like carry-over of substances from the inoculum, or oxygen tension and redox potential (George et al., 1998) before the stressed cells can initiate the growth. It needs to be

pointed out that no-pH-control in shake-flask cultivations entails stressful conditions in addition to the metabolic burden imposed by the requirements for maintenance of recombinant plasmid and antibiotic resistance.

The method of choice for the measurement of dry cell weight (DCW) was centrifugation since filtration is prone to blockage with cell broth before complete drainage of sample. The high r^2 value shows the consistency of the linear correlation between cellular dry weight and optical density of non-induced recombinant *E. Coli* CLD048 (see Figure 3.5, Bottom) and the slope of the regression line falls well within the normal range reported in the literature (0.3-0.6) (Tännler et al., 2008).

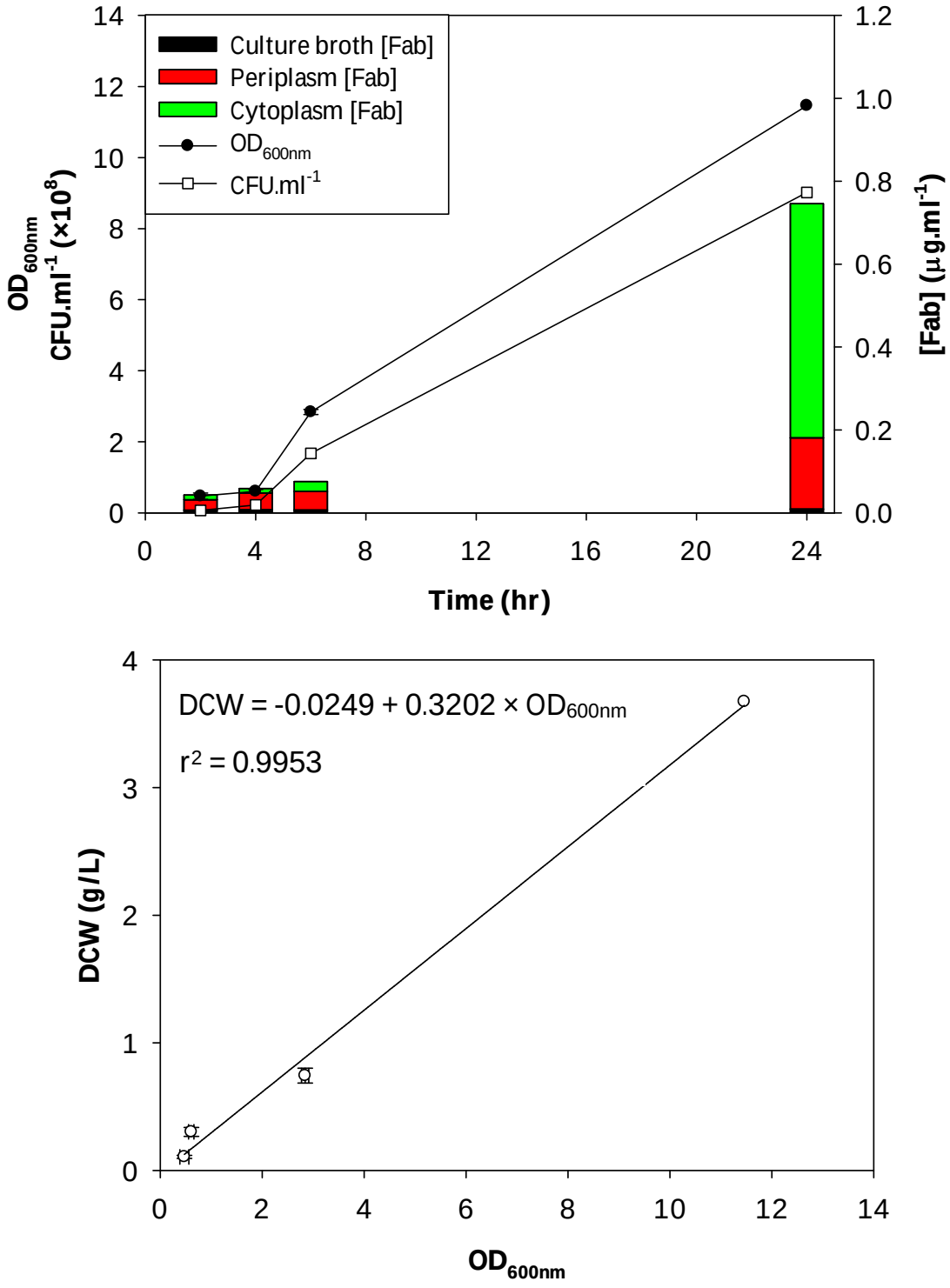

Figure 3.5 Non-induced shake-flask fermentation of recombinant *E. coli* CLD048. Top: Product concentrations in cellular compartments, optical density, and cell number. Bottom: DCW vs. OD_{600nm}. Points plotted are mean values from replicates within the experiment with the errors bars indicating the standard deviation.

Significant decrease in the ratio of DCW to OD has been observed upon induction in recombinant *E. coli* cultures (Vidal et al., 2003; Lewis et al., 2004). Moreover, observations like variations in the ratio of a set of fermentation runs induced with different concentrations of IPTG (Vidal et al., 2005) and a threshold for OD above which the correlation changed (Lewis et al., 2004) signify endangers relying solely on OD for the calculation of biomass in induced cultures. Cellular dry weight measurements were also performed for induced shake-flask fermentations but after little incidents, recalibration of the balance was required which led to inconsistent results.

An early induction detrimentally affected cell growth indicated by a drop in broth turbidity and in viable counts shortly after induction in both of the induced shake-flask cultivations. Upon IPTG addition, the cellular machinery is overwhelmed by the expression of cloned gene product, thus synthesis level of precursors for cell growth is reduced. After induction, cellular demands for metabolites increases rapidly and there is a flux away of nutrients from metabolic pathway towards recombinant protein expression leading to scarcity of carbon/energy source. Furthermore, secretion imposes additional burden on cells since translocation of the premature protein to the periplasmic space requires high energy. In an *E. coli* glucose-limited fed-batch culture, the alterations observed in growth behaviour at a critical specific growth rate inducing severe energy limitations have been correlated to cell segregation into a non-dividing but metabolically-active state and to cell lysis (Andersson et al., 1996a). Segregation into a non-dividing state was observed for plasmid-bearing cells, which was magnified after addition of IPTG without causing disturbance to recombinant protein production (Andersson et al., 1996b). Amongst the genetic programs causing bacterial cell death are

those whose actions are triggered by several stressful conditions including amino acid starvation (Engelberg-Kulka et al., 2006).

The energy limitation imposed on cells by metabolite drain upon induction was intensified by the induction taking place early through logarithmic phase and at a low specific growth rate (the case for both of the induced shake-flask fermentations). It can be suggested that the energy limitation activates the genetic programs leading to cell segregation and lysis. It needs to be pointed out that since the induction took place early logarithmic phase, energy limitation was not caused by scarcity in carbon or nitrogen sources. Instead, it could be correlated to the fact that the cells requirements for metabolites exceed their capability to meet the demands. In comparison, later induction (as for fed-batch fermentations discussed in section 3.1) offers the advantage of an induction time at which the growth and production phase are separated, lowering the requirements of the precursors for cell growth or of the flux of nutrients to the metabolic pathway.

A return to the main phase of cell division was observed for induction with lower specific concentration of IPTG (induction at $OD_{600\ nm}$~1) at the last sampling point though no more protein was synthesized which could be correlated to plasmid loss or some form of structural plasmid instability. The inability of cells to synthesise target protein upon regaining of cultural ability and return of cells to a healthy state as detected by multi-parameter flow cytometry towards the end of fermentation was previously observed (Lewis et al., 2004). That was found to be arising from some form of

structural instability within the recombinant gene sequence rather than plasmid loss since an additional unidentified protein band was revealed by SDS-PAGE gel analysis.

Achievement of lower final optical density for the culture induced earlier (at OD_{600nm} of 0.5) is consistent with the higher dose of IPTG per cell imposing additional burden. The direct proportionality between detrimental effect of IPTG on cell growth and IPTG concentration has been encountered previously (Donovan et al., 2000; Azaman et al., 2010).

IPTG can be transported across the cell membrane with different mechanisms (Pinsach et al., 2008). At low concentrations of IPTG, the only active transport mechanism is the lactose permease, which is produced by *lacY*gene after induction of *lac* operon by IPTG. At high enough concentrations, other unspecified mechanisms become active and participate into the transport of IPTG. Shake-flask cultures were induced with the same volumetric concentration of IPTG as the one induced high cell densities fed-batch cultures (section 3.1) that indicates the higher specific concentration of IPTG imposed on cells in shake-flask cultures rather than fed-batch cultures. Higher dose of IPTG per cell imposes more severe metabolic burden on cells as can be seen from the Fab production profiles of shake-flask and fed-batch fermentations (Figure 3.3 and 3.4). At high concentrations, the quantity of Fab increased sharply after which it reached a plateau or a progressive decrease whereas it increased gradually in case of the induction with low concentrations. This is consistent with the observations by Pinsach et al. (2008) who found a critical value for IPTG concentration (20 μM; corresponding to 153 μmol g DCW^{-1}) in terms of growth rate above which the specific growth rate is highly

diminished. Overloading of host cell metabolism upon pulse induction led to cessation of growth independent IPTG concentration, though inducer concentration determined the length of the production phase.

4. CHAPTER 4 – CONCLUSIONS

Growing *E. coli* cells at the optimal temperature of 37°C followed by temperature reduction to 30°C prior to induction improved the volumetric productivity by almost two-fold as compared with cell growth at the sub-optimal temperature of 30°C (1.7 against 0.85 μg of Fab ml^{-1} h^{-1}). Temperature downshift delayed the basal expression of antibody which brings about higher plasmid stability. Since the two fermentations only differed in the temperature of growth phase, apparently low temperature of growth triggered physiological-changes enhancing protein leakage as substantial antibody leakage to the extracellular milieu ascertained right after induction. Prolonged period of production phase is accompanied by leakage of protein from periplasmic space to the extracellular milieu partly caused by cellular auto-lysis.

The more profound effect of earlier induction on cell growth can be interpreted in terms of specific concentration of IPTG since the volumetric concentration of IPTG was kept constant throughout this study, i.e. higher dose of IPTG per cell impose additional burden on cells. Since production of recombinant protein is growth related, productivity can be highly diminished by overexpression of recombinant protein. Both high biomass concentration and high specific productivity determine the productivity of a fermentation process. Induction at high enough cell density to distinct the growth phase from the production phase and to preclude the cell metabolism overload resulted in longer production phase and higher productivity.

5. CHAPTER 5 – FUTURE WORK

In this study the volumetric concentration of IPTG was kept constant as a value which according to Shibui and Nagahari (1992) falls within the most conducive range for periplasmic expression of recombinant proteins in *E. coli*. The threshold of cellular tolerance to stress produced by overexpression of recombinant protein, which is magnified under substrate limiting conditions, can be investigated in terms of the converse relation between maximum specific production rate and maximum specific growth rate. IPTG volumetric concentration needs to be considered in terms of both tolerant of stress and cell segregation into a non-dividing state which is independent of the critical specific production rate (Pinsach et al., 2008). Moreover, optimum volumetric concentration of IPTG for induction is system dependent as relative amounts of repressor molecules expressed from *lacI* gene is determined by the copy number of both *lacI* gene and promoter in addition to the *lacI* gen itself since for instance its mutant $lacI^q$ produces ten-fold more repressor molecules (Donovan et al., 2000).

Induction with IPTG ended in higher production rates of recombinant in *E. coli* in comparison with lactose (Kilikian et al., 2000) which indicates the severity of metabolic burden on cells under IPTG induction and can be correlated to direct repressor binding of IPTG after being passed through the membrane by either simple diffusion or active transport catalysed by lactose permease. Moreover, higher accumulation of intracellular proteases was observed while the cell culture was induced with IPTG. Addition of lactose with the purpose of induction improved cell growth compared with the non-induced transformed culture while the metabolic load due to expression of Fab fragment triggered by IPTG induction impaired the growth (Donovan et al., 2000).

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