Review of Literature
**The Haemostatic system**

Unless there is any vascular injury, haemostatic system never allows blood to clot. In the case of injuries blood forms a clot (thrombus) to prevent its loss from the body (Fig. 3.1). But sometimes, injuries occur on the inner side of the blood vessels, therefore, blood clots in the lacuna of the blood vessel itself which reduces the room for blood circulation. In severe cases, the clot formed, blocks the flow of blood, hampering normal circulation, leading to serious consequences including death. This phenomenon is very common and is normally observed in the blood vessels supplying blood and oxygen to the heart muscles. The failure of the haemostatic system to dissolve the blood clot quickly often results in stroke, acute myocardial infarction, pulmonary embolism, etc. Treatments require clinical intervention consisting of physical methods like angiography and angioplasty, or the intravenous administration of thrombolytic agents (Collen *et al.* 1988; Francis and Marder. 1991). Streptokinase, t-PA, urokinase and their derivatives are some of the agents developed or are still under development to be used as drug. Staphylokinase is also one such agent having excellent thrombolytic potential (Sherry and Marder. 1991). A number of trials have been conducted for comparing the clinical efficacy of staphylokinase with other thrombolytic agents, revealing a very clear picture of the performance of these agents. Staphylokinase scores much better than the other agents derived from biological sources (Heymans *et al.* 2000; Randall and Collen. 2003, Peter *et al.* 2007).

**3.1. Thrombolysis**

The mechanism and physiology of the formation of blood clot is fairly understood and thoroughly studied (Paoletti and Sherry. 1977). A blood clot (thrombus) consists of enmeshed fibers of proteins mainly fibrinogen and is occluded with blood cells. The body has its own enzyme-based clot dissolving mechanism, which acts on the clot and dissolves it. This is known as thrombolysis or fibrinolysis. In mammalian circulation system, the enzyme responsible for fibrinolysis is plasmin, which is a trypsin like serine protease (Castellino F J. 1981; Rouf *et al.* 1996) and it occurs in micro-quantities in the circulatory system. The fibrinolytically inactive form of plasmin known as plasminogen is present in sufficient quantities in the circulatory system. Conversion of inactive plasminogen into active plasmin involves a limited proteolytic cleavage, mediated by various plasminogen activators (Castellino F J. 1984). Two plasminogen activators that
The Blood Coagulation Cascade

- Intrinsic and Extrinsic pathway of blood coagulation

Extrinsic Pathway

Contact with damaged vessel

Factor XII → Factor XIIa

Fibrin (loose)

Fibrinogen (Factor I)

Factor Va

Factor IX

Prothrombin

Factor X

Factor II

Factor V

Platelet

Phospholipid

Calcium

Factor Xa

Thrombin

Factor XIIa

Factor XI

Factor IXa

Factor X

Factor VIIIa

Factor VII

Calcium

Figure 3.1. Diagrammatic representation of events involved in the coagulation or clotting of blood.
occur naturally in blood are the tissue type plasminogen activators (t-PA) and urokinase type plasminogen activator (u-PA). The modulation of fibrinolytic activity in circulation is mediated by inhibitors of plasminogen activators (e.g. plasminogen activator inhibitor-1 (PA-1), a fast acting inhibitor of t-PA and u-PA) and plasmin (e.g. α2-antiplasmin and β2-macroglobulin) (Francis and Marder. 1991).

Recombinant forms of normal human plasminogen activators t-PA and u-PA are used in clinical intervention. Another commonly used plasminogen activator used in clinical intervention is streptokinase, a bacterial protein that does not occur naturally in human circulation. Staphylokinase is another bacterial protein, which has a promising potential for its use in fibrinolysis. None of these agents possess thrombolytic activity on their own, but their therapeutic action is via the activation of blood plasminogen to plasmin. Unlike t-PA and u-PA, which are proteases, staphylokinase possess no enzymatic activity of its own (Castellino F J. 1981). Staphylokinase acquires its fibrinolytic activation property by complexing with circulatory plasmin. The resulting 1:1, high-affinity stoichiometric complex is a high specificity protease that proteolytically activates other plasminogen molecules to plasmin. Thus, the plasminogen activation mechanism of staphylokinase is fundamentally different from the proteolytic activation t-PA and u-PA

3.2. Staphylokinase (SAK) – The thrombolytic molecule

SAK is a 136-amino acid protein molecule which is produced mainly by Staphylococcus aureus (Fig. 3.2). SAK belongs to the group of staphylococcal proteins, synthesis of which takes place during the late exponential growth phase (Arvidson S. 1983). Four SAK variants with difference in sequence at four amino acid positions have already been identified (Collen D. 1998; Arvidson S. 1983; Collen D et al. 1992'; Sako T et al. 1983; Behnke D and Gerlach D. 1987; Kim S H. 1997). The name Staphylokinase was coined in 1948 (Gerheim E B. 1948), analogous to earlier designation of streptokinase for the plasminogen dependent fibrinolytic activity of Streptococci (Christensen L R. 1945). The thrombolytic potency of staphylokinase was
studied in 1964 and 1985 in dogs. It was found that staphylokinase was poorly thrombolytic, induced severe bleeding and resulted in complete fibrinogen degradation (Lewis et al. 1964; Kanae K. 1985). This is a good example where some observations cannot be extrapolated to humans and vice versa. However, these studies were misleading because the canine fibrinolytic system is unusually sensitive to systemic activation with staphylokinase (Collen D. 1993).

3.2.1. Structure and properties of staphylokinase

The staphylokinase gene encodes a protein of 163 amino acids which is processed into a mature protein of 136 amino acids. Staphylokinase is a single domain molecule (Rabijns et al. 1997) composed of a single polypeptide chain without disulphide bridges. It is predicted to be an elongated dumb-bell shaped molecule (Fig. 3.2; 3.2.1.b). Staphylokinase was found to be structurally similar to the members of β-grasp fold family proteins, according to SCOP protein classification (Murzin et al. 1995). SAK is composed of a mixed five stranded β-sheet (labeled as 1-5) which is packed on a single 12 residue α-helix (Fig. 3.2.1.a). Strand 3, 4 and 5 are adjacent and anti-parallel, as are strands 1 and 2. However, the adjacent strands 1 and 5 are parallel (Richardson. 1976). From 16th residue, staphylokinase folds into a compact, flattened and elongated structure resembling to an ellipsoid. The longest axis of this ellipsoid has a length of 54 Å with other principle axis as 42 Å and 30 Å (Rabijns et al. 1997). The core of the protein is composed of exclusively hydrophobic amino acids. Biochemical studies have uncovered portions of the molecule which are functionally important, e.g. amino acids 46 and 50 are important for complex formation with plasminogen and amino acids 65 and 69 are important for induction of active site exposure in plasminogen. These regions map to the same side of staphylokinase structure. The N-terminal region which is also shown to be important for the activity of staphylokinase is
also situated on this side of the molecule (Wu et al. 2001), which implies that complex formation takes place from this side of the molecule (Silence et al. 1995).

3.2.2. Mechanism of action

Plasminogen activation, mediated by staphylokinase is a complicated process and differs from streptokinase-mediated plasminogen activation. These points can be summarized as:

- The SAK – Plasminogen complex is enzymatically inactive and requires conversion of plasminogen to plasmin.
- Active SAK – plasmin complex is efficiently inactivated by α2-antiplasmin.
- Binding of α2-antiplasmin to the SAK – plasmin complex releases SAK from the complex and makes it ready to interact with other plasmin (-ogen) molecules again (Lijnen H R. 1994; Schlott B. 1998; Okada K. 1996).
- SAK primarily activates fibrin-bound plasminogen (Collen D. 1998) (Fig. 3.2.2.a, b).

The initial step in the activation involves association of SAK with the trace amounts of plasmin formed as the result of weak spontaneous plasminogen activation. SAK – plasmin complex formation is favoured by the 160-fold higher affinity of SAK to plasmin than to plasminogen (Sakharov et al.1996). The primary SAK – plasmin modifies SAK in the complex by cleaving it at the Lys10 - Lys11 peptide bond. This complex subsequently converts SAK – plasminogen to SAK – plasmin, which in turn converts free plasminogen to plasmin. The kringle domains of plasminogen are not involved in the interaction with SAK, and there is evidence that Arg719 in plasminogen and Met26 in SAK are important for binding with plasmin(ogen) (Kim et al. 1997; Lijnen et al. 1994). The NH2-terminal region of SAK is important for the reconfiguration of the active site of the plasmin molecule in the binary complex (Schlott B. 1994). The SAK molecule shows an asymmetric distribution of hydrophobic and hydrophilic residues, which is important for its activation capacity (Jespers et al. 1999).
Substitution of the NH$_2$-terminal charged residues Lys11, Asp13, and Asp14 with Ala results in a mutated SAK that binds plasmin(ogen) but does not convert soluble plasminogen to plasmin. Similar substitutions of Glu46 and Lys50 as well as Glu65 and Asp69 yield mutant proteins with reduced affinity to plasmin(ogen) and subsequently

**Fig. 3.2.2.** Diagrammatic representation of mechanism of action of staphylokinase,

(a). In the absence of blood clot.

(b). In the presence of blood clot

impaired conversion of plasminogen to plasmin (Silence *et al.* 1995). In the absence of fibrin, the formation of the SAK – plasmin complex in plasma and other physiological fluids is efficiently inhibited by $\alpha_2$-antiplasmin, preventing subsequent plasminogen activation. However, inhibition does not take place if the lysine-binding sites of plasmin in the complex are occupied by binding to fibrin or fibrinogen fragments (Okada *et al.* 1996). SAK also binds much more efficiently to fibrin-bound plasminogen than to soluble plasminogen (Sakharov *et al.* 1996) The observation that SAK primarily activates plasminogen bound to fibrin without causing systemic plasminogen activation
has raised great enthusiasm to use SAK as a thrombolytic agent to dissolve the fibrin of blood clots (Collen D. 1998; Christener R B. 1996; Collen D and Van De Werf, 1993).

3.2.3. Advantages of staphylokinase

Staphylokinase has some distinct advantages over the other existing thrombolytic agents.

1. The activation of staphylokinase can be initiated by traces of plasmin, as low as 3 ppm. (Kanae K. 1985; Collen and Van De Werf 1993).

2. Staphylokinase has much lower affinity for native plasminogen than plasmin or for plasminogen bound to partially degraded fibrin (Rabijns et al. 1997).

3. Fibrin has no effect on the activity or activation of staphylokinase (Sakai et al. 1989).

4. In plasma milieu, in the absence of clot, α2 – antiplasmin eliminates free plasmin and inhibits the formation of SAK – Plasmin(-ogen) complex. If any complex is formed its’ rapidly neutralized by α2 – antiplasmin, releasing active staphylokinase molecule. Hence the molecule is recycled and the required dosage is reduced.

5. In the plasma milieu, in the presence of fibrin clot, traces of plasmin are present (due to the physiological plasminogen activation). These plasmin molecules are bound to the clot via lysine binding site and therefore protected against rapid inhibition by α2 – antiplasmin. Hence, staphylokinase is processed locally around the clot and its activity remains concentrated around the clot. Any SAK – plasmin complex if released from the clot into circulation is rapidly inhibited. This makes staphylokinase clot specific and refrains it from systemic generation of plasmin (Fig. 3.2.2.a, b).

6. Staphylokinase is comparably active towards platelet – poor (unretracted) and platelet rich (retracted) clots, whereas streptokinase is only active against platelet poor clots (Lijnen et al. 1992). This property is of much clinical significance as because the high platelet content of a coronary thrombus, together with retraction and ageing, are believed to significantly limit the thrombolytic efficiency of conventional non-fibrin selective agents.

3.2.4. Natural production of staphylokinase

Staphylokinase is mainly produced by Staphylococcus sp., which is the normal flora of the skin, intestine, etc. Staphylococcus is pathogenic, slow growing and requires
stringent precautions while handling this organism, for fermentation studies. At laboratory scale, it produces only 300 μg of staphylokinase per liter of culture broth and the purification yield goes down to 4μgL⁻¹, which is not feasible for large-scale production (Collen and Lijen, 1992). The overproduction of recombinant staphylokinase will be advantageous over the natural strain in terms of product titer and staphylokinase productivity.

3.2.5. Recombinant staphylokinase

Considering the increasing incidences of coronary heart diseases and the therapeutic potential and immense applicability of staphylokinase, attempts have been made in the recent past to produce staphylokinase by other alternative routes, with the principle objective to get high production levels and higher specific SAK expression per unit volume of fermentation medium. A number of systems have been designed and devised for production of staphylokinase.

3.2.6. Secretory production of staphylokinase

In one of the early studies, staphylokinase was extra-cellularly expressed in *Bacillus subtilis* (Lee et al. 2000), by expressing the staphylokinase gene, using a sucrose inducible plasmid. With the optimization of culture and media parameters, the production was attained at 337 mgL⁻¹. The extracellular secretion of staphylokinase was found to be over 90% and was efficiently purified to homogeneity by a simple 3-step purification protocol (Ye et al. 1999). Staphylokinase has been produced from *Escherichia coli* via secretion (Sako et al. 1983; Sako T. 1985) with a production yield as high as 50 mgL⁻¹. Furthermore, micro-heterogeneity at the N-terminal region of secreted staphylokinase caused complications in purification. Some of the secreted staphylokinase molecules were found to miss either 6 or 10 amino acids from the N-terminus residue (Sako T. 1985). Secretory production of staphylokinase by a recombinant *Streptococcus sanguis* Challis 57 strain (Gerlach et al. 1988) has also been reported. *Bacillus subtilis* strains (GB500 and DB 104) that show lower levels of extracellular protease activity has also been applied to produce staphylokinase in the range of 25 – 50mgL⁻¹ (Behnke and Gerlach. 1987).
3.3. Large scale production of heterologous proteins in *Escherichia coli*

With the advancements in recombinant DNA techniques, efficient and accurate control systems and increasing knowledge of genome and proteins, it has become very easy to isolate gene for a particular protein, clone it in an organism with sufficient knowledge of its physiology and induce it for overproduction. *Escherichia coli* remains the choicest host as it's safe, easy to handle, its physiology is well known and proteins can be overproduced even to the level of few grams per liter, that might be difficult, to obtain from any strain that naturally produces the protein. The initial screening and the optimization are normally done at the shake flask level, however, in order to make a process commercially viable; one has to produce it to such a level where it can meet the specifications and economics of an industry. The culture conditions in the shake flask are poles apart when compared to large scale process conditions in fermenters. A fair amount of literature is available on large scale production of many proteins of commercial value using *Escherichia coli* as the host organism. Shake flasks studies have a number of limitations such as low culture volume, no control of pH and dissolved oxygen concentration, inadequate mixing, evaporation losses, etc.

The first and the foremost goal of a fermentation process is to produce the desired protein in the highest possible amounts, in its most active form. Second, this production process should incur minimum losses, investment and equipments. For a culture producing intracellular product, the production can be calculated as (Yee and Blanch, 1992):

\[
\text{Product yield of recombinant protein (per liter)} = \text{Specific expression of the desired protein per unit cell mass} \times \text{Cell Density} \times \text{(g r-protein/g cells)}
\]

Multiplying it with the total volume of fermentation medium will quantify the total amount of protein produced in each fermenter run. This splits the approach of optimization into two parts; the first aim is to increase the specific production of the desired molecule per unit cell mass and second to the increase in the density of cells per unit volume, to achieve maximum volumetric production (Kennedy and Krouse, 1999). Fig. 3.3 shows schematic process of a typical fermentation process along with cell lysis and purification steps.
3.3.1. Modes of fermentation

Fermentation can be categorized in three groups based on the mode of operation;

1. Batch mode
2. Continuous mode and
3. Fed-Batch mode

**In batch process,** all the medium components and nutrients are added to the fermenter prior to inoculation. Once all the components are added, the vessel is closed and inoculated with the desired culture. Nothing is added or removed during the run, except air or mixture of air and oxygen, if the culture is aerobic and requires oxygen for its metabolism. In order to increase cell number and its final density, nutrients are required to be added at their maximum concentrations, but most of the times this initial high concentration becomes itself inhibitory for cell growth and protein production. Hence, batch fermenters don’t generally result in high product concentration. Substrates and nutrients such as glucose >50 gL\(^{-1}\); ammonia >3 gL\(^{-1}\); iron >1.5 gL\(^{-1}\); Magnesium >8.7 gL\(^{-1}\) and phosphorus > 10 gL\(^{-1}\) are shown to be inhibitory (Lee S Y. 1996).

**In continuous mode,** the initial additions of medium components are kept at the optimal level and nutrients are fed while culture broth is simultaneously removed, at specified but equal rates. This avoids the initial buildup of nutrients and subsequent build-up of inhibitory products, if formed. This mode is best for extracellular products (those are secreted outside the cell in the fermentation medium). But for processes where the protein of interest is intracellular, continuous mode of fermentation requires special type of fermenters e.g. fermenters with cell recycling or with dialysis, etc and it also becomes a tedious and cumbersome
process. The fermentation processes that need to maintain specific levels of inducer and antibiotic concentrations in the fermentation medium prove to be quite expensive and also cumbersome in handling. The instability of plasmids and changes in the viability and growth characteristics of the cells in the culture broth after induction, limit the use of continuous cultures in routine.

**Fed-batch mode** of fermentation shares features from batch and continuous modes of fermentations. Fed-batch fermenter is initially operated in batch mode and once the nutrients deplete, fresh nutrients are added at a specified rate, without removing any part of the medium or cells from the fermenter vessel. Once the process is completed, the contents are harvested in the same way as done in the case of batch fermentation.

Batch cultivations of *Escherichia coli* are employed to study the effect of various medium components, e.g. Effect of nitrogen and carbon sources on growth (Bhattacharya and Dubey. 1997), stability of plasmid (Caulcott et al. 1987), formation of acetate (Nancib et al. 1991) and kinetics of cell growth and product formation (Panda et al. 1999). Continuous cultures are highly productive for many microbial fermentations and production processes but they suffer limitations when the culture is inducible with an inducer molecule, e.g. IPTG, plasmid stability, cell viability and change in cell growth characteristics after induction. On the other hand fed-batch processes handle these issues very effectively and are often used to achieve higher cell densities (also known as High Cell Density Cultures; HCDC), thereby improving productivity and minimizing substrate inhibition, dissolved oxygen limitation, toxic by product formation, problems of induction and cell viability (Yee and Blanch. 1992).

### 3.3.2. Fed-batch fermentation

Fed-batch processes are widely used in industrial applications. The fed-batch process begins as a batch process; however, it is excluded from reaching the steady state by starting substrate feed once the initial carbon / nitrogen or an important medium component is consumed. The fermentation is generally continued at a certain controlled growth rate so as to maximize product formation, until some practical limitation inhibits the cell growth. At that point, the feed is stopped and the process is terminated.

### 3.3.3. Feeding strategies in fed-batch fermentation

Fed batch fermentations are normally used to achieve higher cell densities, in comparison to other modes of fermentation. One of the basic needs of fed-batch
fermentation is the feeding of nutrients and their rate of addition into fermenter. Controlled feeding of a limiting nutrient, apart from providing nutrients can also be used to control rates of growth and product formation. Feeding can also be done in a number of ways, namely constant feeding, linear feeding and exponential feeding.

**Constant feeding** is the addition of nutrients at a constant rate, and its addition remains independent of growth or product formation rate. As the cell number increases with time, the feed becomes insufficient to support the increasing nutrient demands of the culture. In this feeding strategy, the volume of the fermentation medium increases, by a constant proportion with time, as the feeding rate is not changed. The specific growth rate of the culture continuously decreases and the rate of increase in the cell concentration also goes down.

**Linear feeding** is the incremental addition of nutrients in the fermentation medium with time. This can be achieved through two modes, gradual increments or stepwise increase in the addition of feed medium. As the cell number increases with time and so the feed rate increases. This increases the amount of nutrients in fermentation medium with time, fulfilling the increasing demands of cells. Hence, the specific growth rate and the rate of increase in the cell concentrations are more efficiently supported and maintained when compared with constant feeding strategies. Higher cell densities can be obtained with linear feeding, when compared to fermentation using constant feeding approach.

**Exponential feeding** mode follows an exponential increase in the feed rate. When the nutrients are present in sufficient concentrations above their limiting concentrations, the microbial cells grow at the highest specific growth rate and their number increases exponentially. Feeding in exponential mode follows the same profile as growth, and the feed is increased in an exponential manner, replicating the exponential growth profile and synchronizing the slope of feed addition with changing slope of the growth curve. The specific growth rate in this mode of fermentation can be maintained at the highest value. The drawback with this kind of feeding is the complexity of the method, requirement of additional equipments and specially designed feed pumps. One of the problems is the generation of by-products such as acetate, which can accumulate to pronounced levels and become inhibitory. It is reported that if the specific growth rate is maintained at 0.1 to 0.3 h⁻¹, the formation of acetate can be easily avoided (Lee S Y. 1996).

**Other feeding strategies** that have been successfully practiced include more complex and sophisticated controls, many times based on the feedback control loops of the
fermenter. The feedback control schemes couple feeding with measurement of various physical parameters such as changes in dissolved oxygen, pH, heat generation, carbon dioxide evolution rate, etc (Lee SY. 1996).

Fed batch fermentation has been an area of constant research and it’s practiced almost in every production process, where there is requirement of higher cell densities. Cell densities as high as 175 g dry cell weight (DCW) per liter have been reported by cultivation of *Escherichia coli* in fed-batch mode coupled with membrane cell recycle or dialysis techniques to remove inhibitory by-products, while the cultures with no dialysis or cell recycling are reported to attain a maximum of 60 g dry cell weight per liter (Landwall and Holme. 1977; Markl et al. 1993). Cell concentrations in the range of 20-40 g dry cell weight per liter (Jeong and Lee. 1999; Obon et al. 1999; Zhang et al. 1999; Ansorge and Kula. 2000) and 50-100 g dry cell weight per liter (Strandberg and Enfors. 1991; Seeger et al. 1995; Horn et al. 1996; Shin et al. 1997; Li et al 1998; Panda et al. 1999; Schmidt et al. 1999; Kweon et al. 2001; Wilms et al. 2001) have routinely been obtained for different *Escherichia coli* strains, with concomitant expression of recombinant protein.

Various research groups have reported different level of recombinant protein expression, but in most high cell density cultivation processes, the highest achieved production ranges from 1 to 3 g L\(^{-1}\). The examples are being the production of Bovine somatotropin (Yoon et al. 1994), Human basic fibroblast growth factor (Seeger et al. 1995), Ovine growth hormone (Panda et al. 1999), interferon alpha (Babu et al. 2000) and human growth hormone (Patra et al. 2000).

### 3.3.4. Factors affecting fed-batch fermentation

Every fermentation process aims to produce the highest attainable amount of production within the domain it operates. The process is also desired to be consistent, robust and should be close to 100% reproducible. It is not only the medium components, feed composition and its rate of addition which must be optimized but equal importance needs to be given to some physiological parameters which need their share of attention to make the process operate and produce optimally. Some of these parameters are plasmid stability, induction conditions and degradation of the target molecule by proteases and dissolved oxygen concentrations.
3.3.4.1. Effect of heterologous protein production on host physiology

Nature has evolved each and every living thing to be most efficient. Hence, the burden laid onto any organism to produce a heterologous protein may result in changes in its physiology or unexpected behavior. A decline in the number of viable cells following the expression of heterologous proteins has been reported by many authors and is assumed to be a consequence of excessive mRNA synthesis (Sugimoto et al. 1987) or product accumulation (Betenbaugh et al. 1989; Kapralek et al. 1991) or reduced availability of carbon source (Strandberg and Enfors. 1991). On the expression of heterologous proteins bacteria have been observed to start growing slowly (Kurland and Dong. 1996). There are reports and experimental evidences that once induced with IPTG, the Escherichia coli cells undergo a lag phase and there is considerable reduction in specific growth rate.

3.3.4.2. Plasmid stability

The probability of the plasmid to retain its existence in the host cell is addressed as plasmid stability. Plasmid stability can be classified into two categories segregational instability and structural instability that are based on its instability characteristics.

Segregational instability is defined as the loss of the complete plasmid from one of the daughter cells due to defective apportioning of the plasmid DNA during cell division. The irreversible multimerization of the recombinant plasmid in Escherichia coli BL 21 (DE3) a RecA+ host has been shown to cause plasmid instability (Summers et al. 1993; Weber and Pal Chaudhury. 1986) and hence result in the decrease of production of recombinant protein (Saraswat et al. 2000).

Structural instability is defined as a change in the structure of the plasmid due to changes in its nucleic acid composition, e.g. insertion, deletion or rearrangement of DNA, which can result in the loss of the desired gene function, while the bulk plasmid in its mutated form, still remains inside the cells with its selective marker intact. Often an antibiotic marker gene is placed on the plasmid to confer resistance to antibiotic like tetracycline, ampicillin, and kanamycin enabling the cells to grow in presence of antibiotics. High concentrations of antibiotics can address the problem of segregational instability but the degradation of antibiotics, their cost and organisms developing resistance against known antibiotics are questionable (Seo and Bailey. 1985*; Ensley B D. 1984). In addition, removal of residual antibiotics from the fermentation medium, as its presence may be unacceptable as a part of product specification.
The number of copies of plasmid present per cell is known as plasmid copy number. The copy number can be as low as one and as high as 500 copies or more. The production of heterologous protein is independent of plasmid copy number, while it can have a significant effect on recombinant protein production, where low copy number plasmids are employed. The strength of the promoter also plays a major role in the production of the target protein. According to Ensley (1984), low copy number plasmids provide a means to optimize productivity by reducing the burden associated with high copy number of the plasmid and allow the use of strong promoters to maximize gene expression. Low copy number plasmids are also used and found to be equally efficient as high copy number plasmids for recombinant protein production (Jones et al. 2000).

3.3.4.3. Degradation of recombinant proteins by proteases

Escherichia coli has at least 24 proteases identified, out of which 12 proteases are localized in the cytoplasm, while others are associated with the membrane (Lazdunski A M. 1989; Harcum and Bentley. 1993). It is practically not possible to engineer an Escherichia coli strain devoid of all proteases. Two defense related cellular responses namely heat-shock and stringent response, induce elevated protease activity. Heat shock response is elicited in Escherichia coli by the presence of recombinant / heterologous protein (Baneyx and Georgiou. 1992), while stringent response is induced by the deficiency of amino acids, resulting from the overproduction of heterologous protein or other nutrient and environmental limitations. Heterologous proteins can also be protected from proteases by inducing them to form inclusion bodies (Tsai et al. 1987; Enfors S O. 1992).

3.3.4.4. Production of inhibitory by-product acetate

Glucose is the simplest and easily assimilated carbon source in nature. Even in presence of other sugars, microbes utilize glucose preferentially, to grow and carry out metabolic processes. Metabolism of glucose results in the formation of a by-product known as acetate which is inhibitory for cell growth and can drastically affect the productivity of a fermentation process (recombinant protein synthesis can also be inhibited if the concentration reached 5 g/L; Shimizu et al. 1991; Yee and Blanch. 1992; Lee S Y. 1996). Acetate formation occurs under conditions of high specific growth rate (Suarez and Kilikian. 2000) and also in situations where the glucose concentration (in the
fermentation medium) is high and dissolved oxygen is low. This results in switching the cells to anaerobic mode of growth (Ko et al. 1994). It happens due to the limited capacity of TCA cycle and the electron transport chain, thereby channeling the flux to acetate formation for the generation of ATP (Han et al. 1992).

A large number of reports have depicted that Escherichia coli B strains produce less acetate as compared to Escherichia coli K strains, under similar cultivation conditions (Kleman and Strohl. 1994; Akesson et al. 2001). This property is attributed to the changes in the metabolic activity of the derivatives of Escherichia coli B strains that leads to reduced rate of glucose uptake and enhancement in acetate utilization using glyoxylate shunt pathway (Kleman and Strohl. 1994; Shiloach et al. 1996; Walle and Shiloach, 1998). Although plasmid stability, plasmid copy number or protease degradation are primarily dependent on the genetic makeup of the plasmid and host strain but these are not independent of the growth environment and cultivation parameters.

3.3.4.5. Pre-induction and post-induction specific growth rate

A variety of correlations have been observed in formation of product and pre-induction specific growth rate, in recombinant cultures of Escherichia coli, producing recombinant proteins. An increase in the pre-induction specific growth rate has been reported to increase the yield of recombinant proteins like β-galactosidase (Sanden et al. 2003) and α-consensus interferon (IFN-αCon1) (Curless et al. 1990), while as opposite effect has also been noticed in cases of β-lactamase (Seo and Bailey. 1985) and human interferon α1 (IFNα1) (Reisenberg et al. 1990) where their concentrations have been reported to decrease when the growth rate of recombinant Escherichia coli increased during fed-batch fermentation. Other workers have reported no correlation between the pre-induction specific growth rate and specific recombinant product formation rate (Shin et al. 1997; Zabriskie et al. 1987). Some authors have advocated the need of an optimum post induction growth rate for optimum production of recombinant protein (Lim and Jung. 1998; Wong et al. 1998; Saraswat et al. 1999).

In all the reports, it has been shown that, growth rate has been varied by the changes in the mode of feeding, the feed composition and feeding rate. If the product formation is related directly to growth, pre-induction feed rate may play a major role. But proteins which are induced at specific time during the process, feeding after induction plays a major role. Genes that are under the control of lac promoter get affected by the presence...
of residual glucose in the fermentation medium. Earlier growth rate was considered to be the major factor affecting productivity and the stability of recombinant plasmids (Lee and Bailey, 1984) while Sanden et al. (2003) have observed that plasmid instability in fed-batch culture is a function of medium composition rather than specific growth rate. Specific growth rate has also been shown to affect acetate production. In fed batch fermentations of *Escherichia coli*, using a semi-synthetic medium, if the growth rate is maintained above a determined specific growth rate ($\mu = 0.30 \text{ h}^{-1}$), acetic acid production was shown to increase (Suarez and Kilikian, 2000). Hence, it is not only the availability of key nutrients in the fermentation medium, but also the rate at which they are made available is important for efficient production of recombinant proteins.

### 3.3.4.6. Effect of dissolved oxygen (DO) concentration

The importance of oxygen for life relates to both its primary use as a substrate and its secondary effects on metabolism. *Escherichia coli*, which is a facultative anaerobe, requires oxygen for maintaining its normal physiological and metabolic state. Aerobic conditions have been reported to support more than 25 times more biomass and volumetric protein yields as compared to anaerobic conditions (Li et al. 1992). Switching from aerobic to anaerobic mode and vice versa changed the rate of synthesis of many proteins by up to 10 folds within a few minutes in fermentation with *Escherichia coli*. These findings suggest that the response to the fluctuating dissolved oxygen concentration is quick and significant at the level of protein synthesis. In addition, dissolved oxygen also has some secondary functions with significant magnitude, e.g. DNA and protein oxidation, plasmid replication, recombinant protein expression and changes in metabolism (Konz et al. 1998). The effect of oxygen on fermentation is generally viewed from two aspects: oxygen transfer rate (OTR) and dissolved oxygen (DO) concentration. Oxygen transfer rate depends on agitation, fermenter design, medium rheology, cell growth rate, etc and changes with change in fermenters. The dissolved oxygen concentration can be easily maintained to a specified value and had been successfully used in many fermentation processes, without much complication (Sahoo and Aggarwal, 2002).

The plasmid stability declines drastically in *Escherichia coli*, going down to as low as 1%, when the aeration was cut off for 30 min during fermentation (Hopkins et al. 1987). The segregational instability of plasmid has been shown to rise rapidly after a step change to low dissolved oxygen tension (DO) in continuous culture of *Saccharomyces*
cerevisiae (Caunt et al. 1989). A change in the rate of aeration has been shown to affect yields of plasmid encoding β-lactamase (Ryan et al. 1989; 1990). Though the plasmid stability went down after induction, the expression of β-galactosidase was not affected (Namdev et al. 1993). There are reports in the literature advocating the requirement of dissolved oxygen for increased production of recombinant proteins. Bhattacharya and Dubey (1997) observed a significant increase (greater than 50 %) in MspI DNA methyltransferases synthesis with an increase in dissolved oxygen level from 0.05 mM to 0.12 mM, in recombinant Escherichia coli with 50 % increase in oxygen utilization rate (OUR) upon induction. Studies done by Li and co-workers (1992) on four different Escherichia coli systems show that the effect of dissolved oxygen concentration on specific expression of recombinant protein was strain specific and dependent on the expression system used. Recently, it has been shown in case of streptokinase production, dissolved oxygen concentration significantly affected plasmid stability thereby affecting streptokinase expression (Goyal D, 2004).

As the fermentation mode shifts to high cell density culture (HCDC), the number of cells per unit volume increase, resulting in the increase in demand for dissolved oxygen, thereby, increasing in overall volumetric oxygen requirement. Since the oxygen transfer capacity is limited for fermenter, the dissolved oxygen often becomes limiting in such high cell density or fed-batch conditions owing to its limited solubility in the fermentation medium. The dissolved oxygen concentration can be increased in the medium by either increasing the overhead pressure (Strandberg and Enfors. 1991), or supplementing the inlet air stream by pure oxygen (Li et al. 1992) or by increasing the aeration or agitation speeds (Jeong and Lee. 1999; Park et al. 1999) or by reduction oxygen uptake rate by culture by controlling the growth rate using appropriate feeding strategies (Clarke et al. 1985; Chen et al. 1992). For Escherichia coli BL21 (DE3) cells, dissolved oxygen concentration above 40 % saturation has been used with pT7 (Saraswat et al. 1999) and pET vector (Shin et al. 1997) systems while dissolved oxygen concentration above 10 % has been used with pAG9 (Wong et al. 1998) with very good expression of recombinant proteins.

### 3.4. The fermentation medium

Productivity of any fermentation process is highly dependent on the development of superior strains through mutagenesis and random screening procedures, as well as optimization of chemical and physical environment to which the organism is exposed.
The fermentation medium determines the chemical or nutritional environment and is thus vital to do research in the area of production of microbial metabolites. Medium optimization has always been the critical component of an industrial and commercial fermentation process, directly affecting not only the productivity but also process economics. Depending on the nature of the material used, the fermentation medium can be classified into three categories: defined, semi-defined and undefined. A defined (synthetic) medium is composed of pure chemicals in precisely known proportions, while a complex (undefined) medium is formulated by including ingredients of natural origin the compositions of which, are not completely known. The term semi-defined (semi-synthetic) refers to a medium composed of mostly defined components with only one or two complex nutrients. The media employed to support high productivity or product yield in industrial fermentations are predominantly formulated with inexpensive complex nitrogen and carbon sources (Miller and Churchill, 1986). As a result, fermentation performance may subject to batch to batch variation inherently associated with ill-defined components. The undesirable variability in the components can sometimes have a major impact on the quality and productivity of the process. One approach to reducing performance variability while maintaining and even enhancing productivity is to replace the complex medium with a chemically defined formulation. Since the components are usually simple chemical compounds and their amounts and structures are known, for many years defined media have been used successfully for microbial / biochemical studies where reproducible results are highly critical. A similar performance consistency is naturally expected when defined media are applied to commercial fermentations. However, many of these simple media require the microbe to synthesize all its cellular components, thus the fermentation performance tends to be poorer than that achieved with complex media, where compounds such as amino acids, vitamins etc. are present as media constituents. When defined media are added with these pure compounds, it becomes quite expensive limiting its use in commercial fermentation processes. To overcome this limitation, one or two complex components are added very often, which are mainly nitrogen sources that provide the cells with the required trace precursors / nutrients and increase the productivity of the process, with low levels of variations.
3.4.1. Advantages of chemically defined / semi-defined media

Chemically defined media are generally preferred in laboratory scale research since they permit one to determine the specific nutrient requirements for growth and product formation by systematically adding or eliminating chemical species from the formulation, with minimal impetus given to the complicated medium interactions. Defined media are thus well suited for fundamental studies of metabolism with better controlled and reproduced culture conditions, but chances might be there that the process may be running at suboptimal levels, resulting in lower productivity and under-utilization of medium components.

It is without doubt that the success of such laboratory studies has helped or could help greatly the success of prospective commercial fermentation processes. However, chemically defined medium is costly as each component is obtained in pure form, which itself is expensive. The cells growing in purely chemical medium are forced to synthesize every cellular component, which generally affects and delays the production of the desired molecule. The lack of vitamin and other complex components required for cell growth and product formation may need these components to be added from outside, increasing the cost of the medium further. In many occasions, delay in growth is observed, resulting in lower cell density and ultimately low productivity, especially in the case of intracellular accumulation of product.

3.4.2. Fermentation with chemically defined medium

Although complex medium formulations continue to dominate the fermentation industry because of lower cost and more robust cell growth, due to the presence of many vitamins, minerals and macromolecular precursors, there are occasions where encouraging levels of secondary metabolites are achieved when relatively simple and inexpensive defined media are employed on a large scale (Zhang et al. 1996). In fermentations like those for steroid bioconversion as the products are of relatively high-cost, yields become the predominant consideration; thus high-yielding chemically defined media are used in some cases (Miller and Churchill. 1986). For the production of ice-nucleating activity from Pseudomonas syringae, Ward et al. (1989) reportedly scaled-up a defined-medium process to a ‘commercial scale’. Defined media have become more popular for the production of biological products, including recombinant
proteins, on the industrial scale (Zhang et al. 1998; 1999), especially when optimized to achieve production levels equal to or greater than those achieved with complex media. An almost double xylitol production yield by a *Candida guilliermondii* strain was obtained in a synthetic medium compared to a sugarcane bagasse hydrolyzate medium (Pfeifer et al. 1996), because of the accumulation of various toxic substances (e.g. acetic acid) in the complex medium that interfered with microbial metabolism. In the 500-l pilot-scale *Aspergillus niger* fermentations, a sucrose based synthetic medium supported a citric acid yield equal to or better than that obtained in two complex media (Qazi et al. 1990).

### 3.4.2.1. Enhanced process consistency

As compared to complex media, chemically defined media inherently support a more reproducible fermentation performance, an important and desired characteristic for any industrial fermentation process. In a fermentation process of recombinant yeast producing a therapeutic protein (Zhang et al. 1998), extremely consistent results in biomass and protein production were obtained in over 25 batches on a 23-l fermenter scale, and superimposed fermentation profiles were obtained in consecutive pilot-plant 1000-L batches spanning 6 months.

### 3.4.2.2. Better control and monitoring

The ability to characterize a chemically defined medium offers the potential for maximal process control and monitoring. In a fed-batch *Saccharomyces cerevisiae* fermentation on a 20L scale (O' Connor et al. 1992), the use of defined medium allowed the precise control of carbon source concentration through multiple strategies, achieving high cell densities of 79 g dry cells/l and yields of 0.50 g dry cells/g glucose consistently.

### 3.4.2.3. Improved process scale-up

Understandably, defined media have been reported to be much less sensitive to sterilization conditions than complex medium formulations (Jain et al. 1992; Zhang et al. 1998), thus minimizing a major scaling-up problem. In addition, defined media are usually highly soluble and give consistent compositions, in spite of possible differences in the origins of the ingredients and mixing conditions at various scales. Consequently, fermentation processes can often be scaled-up faster than those with complex media. In some fermentations, such as that for biotin production by recombinant *Escherichia coli*,

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the optimization and scaling-up of the process with complex medium containing casein hydrolysate were considered difficult and costly; this problem was relieved by the development of a defined medium, as only two amino acids, histidine and methionine, were actually required for growth and optimal production (Bonsignore et al. 1989).

3.5. Development of chemically defined / semi-defined medium

Cell growth and product formation in fermentation may be regarded as a stoichiometric relationship, in which carbon / energy source, nitrogen source, minerals and oxygen are converted to biomass, products / by-products, carbon dioxide and water. When chemically defined medium is used, the microbe must synthesize all cellular components as well as products / by-products from simple, chemically defined substrates. Thus, initial medium formulation usually focuses on the cellular composition, cell physiology and desired cell concentration of the microbe of interest. Once the components are standardized, usually complex components are added in order to fulfill the requirement of the culture for vitamins and other trace components. Published defined media for the related microbes and previously used complex media with the complex nutrients can be considered as a base for further manipulation of medium composition.

Addition of complex nutrients such as tryptone, yeast extract, peptone, and casein hydrolysate often help the organism to grow and improve the quality and amount of the protein produced. These complex nutrients act as a source of amino acids, vitamins and other biosynthetic precursors. There is a plethora of information, in literature, advocating significant increase in production yields (approximately ten folds), of recombinant proteins by manipulating medium composition. Some authors have attributed the increase in the expression level of recombinant protein with addition of organic nitrogen sources to reduce burden on the cell due to availability of biosynthetic precursors (Zabriskie et al. 1987; Kweon et al. 2001). Others have described the increase in the expression of recombinant protein due to the suppression of protease activity by amino acid present in these complex nutrients (Mizutani et al. 1986; Tsai et al. 1987; George et al. 1992). In addition, complex nitrogen sources have been reported to increase the plasmid stability (Matsui et al. 1990) and also improve the copy number of the plasmid (Shin et al. 1997). At the same time, the inhibition of protein synthesis beyond a certain concentration of complex nitrogen source is reported (Rinas et al. 1989; Matsui et al. 1990; Li et al. 1998).
Once growth and the synthesis of the desired product is achieved, the medium usually requires optimization to achieve economical performance. The development of an optimized medium is often time-consuming, when optimization is approached using one component at a time approach. Quicker optimization may be achieved by employing statistically designed experiments. Methodologies that have been successfully employed in the development of complex media, such as an expert system approach (Kishimoto and Suzuki. 1995), Plackett – Burman Design, Response Surface Methodology, etc. certainly have great potential to be applied successfully to the development of a robust and productive medium.

### 3.6. Optimization of fermentation medium

The optimization of fermentation medium can be done by a number of approaches reported already in the literature. Broadly, the method can be divided into two classes, classical (one at a time) and statistical approach. Medium optimization generally refers to determining the appropriate nutrients and establishing the concentrations that will support the best cell growth and or synthesis of a particular microbial product. However, optimization should be attempted within the context of the overall process requirement.

#### 3.6.1. Classical method of medium optimization

Traditionally, medium optimization with defined components is conducted in a sequence of shake-flask experiments. A change in the concentration or nature of medium components is made and the resulting change in growth rate, cell density and or product yield is compared to that of the previous experiment. By using a ‘single-omission’ technique, Cocaign-Bousquet et al. (1995) determined the true nutritional requirements and greatly simplified their synthetic medium for the sustained growth of *L. lactis*. El Kady and Moubasher (1982) evaluated a number of carbon and nitrogen sources and amino acids one by one, and prepared a properly defined medium for the production of verrucarcin, a cytotoxic metabolite of *Stachybotrys chartarum*. Other successful optimizations using the single-factor approach have also been reported (Monot et al. 1982; Chary et al. 1989). But, fermentation or the culture medium is not a formulation where the effect of every component is isolated from the other. There is considerable interaction of one component with the rest. However, microbes too behave in a very complex way with respect to the uptake and utilization of medium
components. One component may be preferentially utilized over the others; a component might suppress the effect of by-products released by the organism, diminishing concentration of one component may switch the preference of the culture for another component. Classical methods do not account for such kind of interaction of medium components and also do not accurately show the absolute effect of one component on growth or other parameters under consideration. In addition, such methods, although simple, often require considerable work and time.

3.6.2. Statistical method of medium optimization

An alternative optimization strategy, which has become popular especially in industry, is the use of statistically designed experiments that allow the investigator to evaluate more than one independent variable at a time (Greasham and Inamine, 1986; Greasham and Herber, 1997). The approach generally starts with screening to distinguish those variables (normally three to five or sometimes more depending on the culture requirement) that have a significant effect on the desired response from a larger number of potential variables (more than five) with a minimum of testing, employing fractional factorial designs such as the Plackett-Burman method (Plackett and Burman 1946; Roseiro et al. 1992). Optimization of defined media by the Plackett-Burman design was reported by many workers (Metzger et al. 1984; Kisaalita et al. 1993; McIntyre et al. 1996). A full factorial search is generally impractical for medium optimization because of the huge number of trials involved, unless only a few variables (between two or three) are to be examined (Garcia-Ochoa et al. 1992). The next step is usually to find the combination of these variables that supports the best acceptable response in a timely manner, using a response-surface type of design, such as a central composite or Box-Behnken design. Examples of the successful use of surface-response designs have been reported (Zhang et al. 1996). In the fermentation of C. bombicola, which produces the industrially important surfactant sophoro-lipid, Casas et al. (1997) first employed a four-factor, two-level factorial design followed by a three-factor, three-level surface-response design to optimize a synthetic medium. The curvature-effect analysis revealed that magnesium had no significant effect while nitrogen and phosphorus sources achieved maximum values for culture growth. Other workers have used RSM for biosorption of chromium (Margarita et al. 2005), ethyl butyrate production by lipases (Jose et al. 2005) Another easy-to-use optimization method is the sequential simplex method (Spendley et al. 1962; Leggett et al. 1983), the optimum area is approached
through a series of sequential steps toward better results. The maximal value can be obtained with a minimal number of steps. This pattern-seeking approach is non-statistical, but is quite useful in identifying the experimental optimum rapidly.

3.7. Economics of using chemically defined media in fermentations

At commercial scale fermentations, economics usually plays a key role in medium development. Often, low product yield and the high cost of defined media make complex or natural media the preferred choice, especially for low cost products like antibiotics and bulk chemicals. The latter media consist of raw ingredients that are usually inexpensive, abundant, and readily available. While certain microbes, e.g. *Streptomyces*, can grow well in simple defined media, others practically can’t. In comparison with complex media, the need for large numbers of additives to defined media (amino acids, vitamins, growth factors, etc.) has often discouraged the attempt to develop defined media for industrial use. Use of synthetic medium in the microbiological production of inosine using *Bacillus subtilis* B-1312 strain (Erokhina and Kazarinnova. 1982) requires addition of adenine, tyrosine and histidine. In some cases, despite these additives, a slower growth rate and/or low cell density were still experienced when defined medium was used to replace complex medium, such as in some strains of recombinant *Escherichia coli* (Chou *et al*. 1994), B. subtilis (Leitch and Collier. 1996), *L. lactis* (Tanaka *et al*. 1995), and *Streptococcus pyrogenes* (Dassy and Alouf. 1983). Compared to bacteria, yeasts and fungi are generally less fastidious, but vitamins are still frequently needed for the growth of these cultures (Bacon. 1985). Because of the slow growth defined media, fermentations tend to have longer cycle times which lead to less impressive productivities (titer in unit time). Hence, addition of complex components in growth and production medium will remain a definite choice for large scale industrial fermentation.

3.8. Haemoglobin – The oxygen carrier

Oxygen is required for the regulation of a variety of cellular functions. Apart from respiration, oxygen can directly or indirectly affect bacterial cells in at least two ways: altering the function of specific proteins or affecting the biosynthesis of specific sets of proteins. The latter effects are largely due to the effects of oxygen on the function of global transcription factors.
In humans, haemoglobin (Hb) is the protein molecule that is associated with the utilization of oxygen. Recent findings indicate ubiquitous existence of haemoglobins in mammals, non-vertebrates, plants, and bacteria. The knowledge concerning bacterial globins and their role in cellular metabolism is a subject of increasing basic and applied research.

The common characteristic of all Hb molecules, encountered in all five kingdoms of life, is their ability to reversibly bind oxygen. Alignment of the amino acid sequences from almost all sources, reveal a highly variable or even almost random primary amino acid sequence, two key residues are conserved in all globin proteins encountered: Phe at position CD1 (Perutz, 1979) and His at position F8. Analysis of protein structures of globins reveal a typical tertiary structure, known as the classical globin-fold.

The obligate aerobic bacterium *Vitreoscilla* synthesizes a homodimeric Hb (VHb) (Webster and Hackett. 1966) which is one of the best-characterized members of bacterial Hb protein family. The *VHb* gene encodes an oxygen-binding protein of 15.7 kDa (Wakabayashi et al. 1986; Dikshit and Webster. 1988; Khosla and Bailey. 1988\*; 1989\*). Studies on the localization of VHb expressed in *Escherichia coli* have revealed a distribution of the protein into both the cytoplasmic and periplasmic space, with up to 30% of the active protein being found in the latter (Khosla. and Bailey. 1988\#; 1989\#). Recently, VHb localization using electron microscopy in *Vitreoscilla* and *Escherichia coli* indicated that VHb has mainly a cytoplasmic and not a periplasmic localization in both organisms suggesting the periplasmic localization of VHb due to the overexpression (Ramandeep et al. 2001).

**3.8.1. Regulation of VHb expression**

The oxygen-dependent promoter of *VHb* (pVHb) is induced under oxygen-limited conditions in *Vitreoscilla* (Webster and Hackett. 1966). pVHb has been characterized in *Escherichia coli*, and has been shown to be functional in various heterologous hosts, such as *Pseudomonas*, *Azotobacter*, *Rhizobium etli*, *Streptomyces sp.*, *Serratia marcescens* and *Burkholderia sp.* (Khosla and Bailey. 1988\*; Dikshit et al. 1990; Magnolo et al. 1991; Wei et al. 1998\*; 1998\#; Patel et al. 2000). pVHb is maximally induced under micro-aerobic conditions in both *Vitreoscilla* and *Escherichia coli*, when the dissolved oxygen level is less than 2% of air saturation (Khosla and Bailey. 1988\*). Activity of pVHb in *Escherichia coli* is positively modulated by CRP and FNR. Expression from pVHb is substantially reduced in strains that are unable to synthesize
CRP or cAMP. Supplementation of cAMP increased the expression level 10-fold in cya mutant strains (Khosla et al. 1990).

3.8.2. Biochemical function of VHb

Upon expression of VHb in *Escherichia coli* under oxygen-limited conditions, recombinant cells grew to higher final cell densities relative to control cell (Khosla and Bailey. 1988). Further experiments showed that the effect of VHb was not restricted to growth improvements, but also resulted in enhanced protein synthesis (Khosla et al. 1990). Tsai et al. (1996) showed the beneficial effects of VHb in *Escherichia coli*, correlated with elevated VHb expression levels. Khosla et al. (1990) proposed that VHb expression could facilitate oxygen diffusion and improve aerobic metabolism in *Escherichia coli*. Due to the observed interplay of VHb with terminal oxidase, VHb might affect the electron flux through the respiratory chain in *Escherichia coli*, thus changing the NAD(P)+/ NAD(P)H ratio and increasing primary carbon metabolism. The responses to rapidly changing oxygen tension were slower, suggesting that VHb buffers the intracellular redox state by sustaining normal respiration during the absence of aeration (Tsai et al. 1995). Furthermore, VHb-positive cells contained increased amounts of tRNA's and active 70S ribosome complexes under micro aerobic conditions in *Escherichia coli*. This was also accompanied by a corresponding increase of a marker enzyme activity (Nilsson et al. 1999).

3.8.3. Use of VHb to improve cell growth and productivity

To alleviate adverse effects of oxygen limitation on microorganisms, molecular biology approaches are sought (Bailey. 1991). The approach is based on previous observations that *Vitreoscilla* expresses Hb under oxygen deprivation. The *VHb* gene was successfully transferred to *Escherichia coli* and upon expression of VHb, growth and protein production of *Escherichia coli* was enhanced under micro aerobic conditions (Khosla and Bailey, 1988). The positive effects of *VHb* expression, promoting either the efficiency of oxygen-limited growth and / or production of primary and secondary metabolites in numerous microorganisms, are well documented in literature. VHb has pleotropic effect on cellular metabolism and it can affect more than one cellular biochemical process, e.g. energy metabolism, oxygen uptake, electron transport, etc. The beneficial effect of VHb may be due to the combined effect of VHb. This technology seems to be especially beneficial for mycelium forming microorganisms,
e.g. antibiotic-producing *Streptomyces* strains, *Acremonium crysogenum* and *Saccharopolyspora erythraea*, etc. It is known that oxygen transfer to the mycelial pellet is limited and is especially critical when such microorganisms are grown in highly viscous industrial media. In such situations, VHb expression seems to enhance antibiotic production. The VHb expression technology has been successfully tested, using an industrial *S. erythraea* strain producing erythromycin (Brunker *et al.* 1998). VHb expression significantly increased yield of erythromycin production to approximately 100% relative to the original strain (Minas *et al.* 1998). VHb expression has been shown to be useful in improving the bioremediation capacity of *Burkholderia* sp. strain DNT and indeed expression of VHb improved the rate of 2, 4-DNT degradation (Suen and Spain. 1993; Patel *et al.* 2000).

### 3.9. Downstream Processing – Cell lysis and Protein purification

After the successful production of recombinant proteins, the next essential step is the recovery of protein in its active form, with maximum yield, minimum investment, equipments and less number of required steps. Downstream processing (including cell disintegration and purification) is a very critical process where even the slightest sub-optimal operations may lead to reduced recovery of the protein of interest and may result in higher capital investment. The choice of the cell lysis and protein purification process mainly depends on the nature, localization of the protein of interest, intended use of the product, stability, solubility and nature of the protein. When the protein is secreted outside the cell, protein concentration methods, e.g. precipitation, ultrafiltration, etc are employed. While intracellular localization of protein requires cell separation from fermentation broth, mainly done by centrifugation and subsequently followed by cell lysis to recover the protein. Once the protein is concentrated to the desired level, real purification procedures are followed. These are mainly ion-exchange, hydrophobic interaction and sometimes gel filtration chromatography as a final polishing step. The purity of the protein is pre-requisite for its structure and function studies and also for its potential applications. The required level of purity mainly depends on the intended end use of the product, a high degree of purity is needed for therapeutic purposes and structural studies and comparative lower degree of purity is required for industrial application, such as food industry, detergent, cosmetics, leather industry etc. The global aim of the purification process is not only to remove the unwanted contaminants but also the concentration of the desired protein and their
transfer to an environment where it is stable and in a form ready for the intended application of the protein.

3.9.1. Methods of cell disruption and its quantification

Disruption means breaking apart or interrupting anything, leading to disorderliness. This interpretation can be easily extrapolated to the disruption of microbial cells that means the breaking of cell wall to release the intracellular product, easing the recovery and purification, when the desired protein is located intracellularly (Abrahmsen et al. 1986; Hsiung et al. 1989; Kato C. 1987; Yu and San. 1992). For an industrial scale process, efficiency and productivity are major and critical parameters, which are considered, too while designing the disruption process (Fish and Lily. 1984; Schein C H. 1989). Disruption can be measured by a number of means; the simplest measure of the disruption is the fraction of cells destroyed, in terms of volume or number. This includes methods which can be divided into direct and indirect. The direct methods involve direct measurements of disruption and can be quantified by simple methods like microscopy which takes into account simple staining to identify the loss of cell wall integrity. This methodology lacks the precision and its time consuming. To overcome these limitations, methylene blue dye exclusion assay was employed with automatic cell counting, using a Naubauer chamber or hemocytometer (Melendres et al. 1992; 1993). A direct measure of disruption is not possible due to difficulty in separation of cells from the cell debris.

Indirect methods do not take into account the breakage of cell integrity per-se; instead they seek the quantitation of released intracellular products. The easiest and most common approach is the measurement of total cell protein in sample supernatant using the Bradford (Bradford M M. 1976) or Lowry (Lowry et al. 1951) or BCA (Smith et al. 1985) protein quantification assays. The fractional protein release ($P_f$) for dilute samples can be given by the equation (4.1.a),

$$P_f = \frac{C_i - C_i}{C_m - C_i}$$  \quad \text{Eq.a.1.}

Where

$C_i$ is the concentration of protein in the disrupted supernatant

$C_i$ is the initial concentration of protein in the sample

$C_m$ is the maximum possible concentration of protein in the supernatant
If there is no initial concentration of protein in the suspension, the equation simplifies to

\[ P_f = \frac{C_f}{C_{mi}} \]  

Eq. a.2.

Hence, the disruption efficiency of the process can be given by the ratio of the protein released in the process, to the total amount of protein present. But when the concentration of cells in the disruption medium is high, volume correction has to be made as the aqueous volume fraction increases during disruption.

The choice of method depends on the process parameters and the intentional use of the product released. Indirect methods are generally preferred as they are simple, quick, inexpensive, and less labor oriented. Further, if the aim is to recover a specific product, then a direct quantification of the product will give the amount or the extent of cell disruption. For optimization / modeling a specific disruption process, direct methods are more accurate and yield best results.

3.9.2. Classification of cell disruption processes

If the cell wall structure of the organism under study is known, it becomes easy to devise a disruption process, without doing any harm to the product of interest. Laboratory scale cell disruption methods have also been reviewed extensively (Hughes et al. 1971). These include French press (Morein et al.1994; Schmitt B. 1976), agitation with glass beads (De Virgilio C, 1991) sonication (Bunge et al. 1992; Feliu J X and Viilaverde A. 1994; Morein et al. 1994), etc. At larger scale, mechanical methods are more practical and efficient. Hence, the disruption

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**Fig. 3.9.2. Classification of cell disruption methods**
processes are restricted to bead milling, high pressure homogenization and microfluidization. The flowchart in (Fig. 3.9.2) summarizes the disruption methods.

The disruption methods can be categorized as mechanical and non-mechanical. Mechanical methods are harsh, can be used at large scale and handle large amount of cells unlike non-mechanical methods. Non-mechanical methods can further be subdivided into physical, enzymatic and chemical methods. The physical methods rely on the disruption of the wall structure without tearing it entirely apart, e.g. decompression relies on rapid expansion of gas within the cell and often results in point puncture of the cell. Chemical methods rely on selective interaction of a chemical compound with components of the cell wall. Disruption in this way allows the product to sweep through the peptidoglycan layer. If the protein of interest is situated within the periplasmic space, chemical attack on the outer membrane results is higher releases. Enzymatic lysis also depends on the dissolution of the peptidoglycan layer in gram-negative bacteria like *E. coli*. The outer membrane renders the protection against the enzymatic attack in *E. coli*. Therefore, enzymatic treatments are normally coupled with chemicals that disrupt the outer membrane, allowing the access of enzymes to the peptidoglycan layer (Johnson and Hecht. 1994; Kwan *et al.* 1991). Additional methods include freeze grinding and repeated freezing and thawing. It should be taken into consideration that sometimes the classification is not so accurate or precise.

3.9.2.1. Physical disruption methods.

There are a limited number of physical disruption methods available. But these methods possess a higher potential, for cell disruption at a larger scale.

3.9.2.1.1. Decompression

When a cell is kept under a sub-critical or supercritical gas with high pressure, the gas enters the cell. This results in pressure equilibrium inside and outside the cell. After certain time, the overhead pressure is released very quickly. Since the pressure drop is quick, just like an explosion, the gas inside the cell expands (Lin H M. 1991; 1992). The cell wall being unable to contain the force from inside, expands and ultimately gets perforated at places due to the escaping gas. *E. coli* has been disrupted at laboratory scale using this technique (Fraser D. 1951). This method is gentler, results in large debris, which is easy to remove, where the desired product is soluble. Results highly
depend on the time of contact between the gas and suspension and the rate of pressure release.

3.9.2.1.2. Osmotic shock

The technique resembles decompression, and the principle behind it remains nearly same. The cells are first equilibrated with a medium of high osmotic pressure (e.g. 1 M sucrose), and then suddenly diluted. During equilibration, the water content of the cells decreases and the cells shrink. On rapid dilution, water enters the cells, increases the internal pressure and causes the cells to burst, releasing the inside contents. The technique is advantageous for thermally unstable proteins, but it’s restricted to weakened cells (Hughes. 1971), as its not capable of disrupting the strength providing components of the cells, mainly gram-positive cells which have cell walls rich in peptidoglycan (Nen and Heppel. 1965). The process is not considered for large-scale disruption process owing to the high cost of additives and increased biological oxygen (BOD) demand of the process waste.

3.9.2.1.3. Thermal lysis

Gram-negative bacteria such as *E. coli* when heated to 50-55°C, the outer membrane disrupts releasing the periplasmic proteins (Katsui *et al*. 1981; Tsuchido *et al*. 1985; Watson *et al*. 1987). Results in this method are dependent on the type of organism used in the study and its growth phase. The gram-positive bacteria like *Bacillus megaterium* gives low intracellular protein release at high temperatures. The protein released from the cells of *E. coli* and *B. megaterium* was found to be higher when lysed at stationary phase as against exponential phase (Watson *et al*. 1987). Results also depend largely on the storage temperature prior to cell lysis. The protein release reduces when the cells are kept at low temperatures (e.g. 0-4 °C) and makes them more resistance to thermal lysis. This results due to the change in the fatty acid composition of the cells, e.g. *E. coli* and *A. eutrophus* (Katsui *et al*. 1981).

The technique offers distinct advantages at the process-scale. Firstly, it can be used to kill the host organism, reducing the chances of possible release of the organism. Preferential enzyme inactivation / degradation (e.g. proteases) can be achieved if the product is thermally stable. Removal of cell debris is easy when the product is soluble. This process cannot be used if the desired product is thermo-labile. When the products form inclusion bodies further separation becomes tedious. Mild heat treatment designed
to kill the host may result in smaller and toughened cells, thus making subsequent disruption processes less efficient (Vogels and Kula. 1992; Coillos et al. 1995).

3.9.2.2. Chemical disruption methods

The outer wall of microorganisms can be disrupted or permeabilized by a variety of chemical treatments (Naglak et al. 1990; Hancock R E W. 1984; Felix H. 1982). The primary methods are discussed below.

3.9.2.2.1. Antibiotics

As stated earlier, the growth phase of the cell has a great influence on the selection of cell disruption method and also on the lysis efficiency. Antibiotics are particularly effective for lysing the bacteria e.g. *E. coli*, in the active state of its growth. A range of antibiotics is available, with each having its own particular method of lysing the cell. The most common class is the β-lactam antibiotics that affect peptidoglycan synthesis or by incorporation as a result of interaction with penicillin binding proteins (Kohrausch and Hiltje. 1991). Lysis is believed to occur as a result of splitting of the peptidoglycan by exo-muramidases, restricted to the equatorial zone following the peptidoglycan precursor synthesis. Without peptidoglycan, the cell is unable to maintain its osmotic pressure and consequently disrupts. A cationic polypeptide antibiotic, known as Polymyxin, disorganizes and penetrates the outer membrane and binds and distorts the cytoplasmic membrane (Nikaido and Vaara.1985). The efficiency of the process is confined to smaller scale and there have not been reports on the use at process scale. This might be due to the high cost involved and dependency of the efficiency of the process on the growth phase of cells.

3.9.2.2.2. Chelating agents

Chelating agents such as ethylenediamine tetra acetate (EDTA) can disrupt the outer membrane of gram negative bacteria by binding divalent cations such as Mg$_{2}^{+}$ and Ca$_{2}^{+}$. These ions form a cross bridge in between the lipopolysaccharide molecules resulting in the release of lipopolysaccharide patches containing protein and phospholipids (Leive et al. 1968). The space created by this release is rapidly filled up by phospholipids from the inner leaflet of the membrane (Nikaido and Nakae. 1979). The release is quick and its independent of the operating temperature, but dependent on the culture. However, EDTA is not very effective in release of cytoplasmic proteins or enzymes, because it
has virtually no effect on the inner membrane which remains intact even after EDTA treatment (Nen and Heppel. 1964; De Smet et al. 1978; Hancock R E W. 1984).

3.9.2.2.3. Chaotropic agents

Chemical agents such as Urea and Guanidine hydrochloride, when added to water reduce the hydrophilicity and disrupt the ordering of water molecules, thus making the solute-solute interaction weak (Naglak et al. 1990). The hydrophobic membrane proteins may be solubilized in sufficiently concentrated and effective chaotropic agents. Recovery of membrane proteins from *E. coli* was done using guanidine thiocyanate (Moldow et al. 1972), with 80% efficiency. Ethanol, on the other hand, is an effective chaotropic agent. Addition of ethanol disrupts hydrogen bonding in water and also hinders the assembly of cross-linked peptidoglycan, subsequently resulting in lysis (Flores et al. 1994; Ingram L O. 1981). The large scale use of chaotropic agents for cell disruption is not reported. This is partly due to the variation in efficiency with the target organism and its growth state and other physical parameters like temperature, concentration etc. Also, the high concentration of chaotropes required confers handling problems, high cost and also expenses for its removal from the final product and waste disposal.

3.9.2.2.4. Detergents

Detergents are amphipathic molecules having a hydrophilic and a hydrophobic portion (Belter et al. 1988; Helenius and Simons. 1975), therefore they are able to interact with both water and lipid. Surfactant bind to the lipid membrane till saturation and further addition of surfactant leads to the formation of mixed micelles until the entire lipid is contained in the mixed micelles. Addition of surfactant beyond this point leads to the formation of surfactant micelles (Jones, 1992). A range of surfactants are available e.g. anionic (SDS, salts of fatty acids, etc), cationic (tetra-alkyl ammonium salts and non-ionic (Triton X, Brij series). Though, the process is mild and generates cell debris that are larger in size and hence easy to separate from the released proteins in the solution, they suffer from a drawback of their removal from the protein solution or the lysate. Detergents are useful for recovering membrane proteins from gram negative cells but the high concentration of detergents add to the cost of the process and further complicate the downstream processing.
3.10. Large scale cell disruption: The Bead Mill

Bead mills provide a simple and effective means for disrupting microorganisms, although they were originally designed for the wet-grinding of pigments for the paint industry (Hopkins T R. 1991; Kula and Schitte. 1987) and for the fine grinding of ceramics and limestone (Bunge et al. 1992). Various designs of bead mills are available. The basic approach is to have a jacketed grinding chamber with a rotating shaft through its centre. The shaft is fitted with agitators that impart kinetic energy to small beads in the chamber, forcing them to collide with each other. The beads (typically < 1.5 mm glass) are retained in the grinding chamber by a sieve or an axial slot smaller than the bead size (MacNeil et al. 1985). The process generates a lot of heat, hence there is always a need for cooling the chamber. Cells are believed to disrupt in the contact zones of the beads by compaction or shearing action (Bunge et al. 1992; Melendres et al. 1991; 1992) and by energy transfer from beads to cells (MacNeil et al. 1985), although an exact understanding of the mechanism is not presently available. Disruption is highly non-specific as the cell wall is destroyed by the tearing apart of the cell wall components. It can be seen that good starting conditions to maximize disruption for any particular application are high bead loadings (70-80% of free grinding chamber volume), relatively high agitator speeds (peripheral velocity 5-10 m s⁻¹) (Dunnill and Lily. 1975), small bead diameters (0.5 mm), and moderate to high cell concentrations (40-50% wet weight) (Chisti and Moo Young. 1986; Engler C R. 1985; Kula and Schitte. 1987). The effect of cell concentration seems variable, and may be attributable to changes in broth rheology (Garrido et al. 1994). The release of D-glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, was maximized with beads of intermediate size (0.55-0.85 mm). Conversely, the release of β-D-glucosidase, which is predominantly located in the periplasm, was maximized with larger beads. This is explained by the fact that complete disintegration of the cell is not necessary to release periplasmic enzymes. This has been also confirmed that the rate of enzyme release is dependent on the location in the cell (Torner and Asenjo. 1991). Batch studies on
Saccharomyces cerevisiae showed maximum release of invertase, D-glucosidase, alcohol dehydrogenase (ADH) and fumarase after 2, 5, 10 and 15 minutes of disruption, respectively. The first two enzymes are located primarily outside the cytoplasmic space and are therefore released faster than ADH (located in cytosol) and fumarase (located in mitochondria). The release of invertase, a wall-bound enzyme, is also faster than the release of cytoplasmic enzymes during bead milling (Melendres et al. 1993, Van Gaver and Huyghebaert. 1990).

Temperature rises in bead milling can be quite substantial at high speeds or when the mill is scaled up due to the large amount of energy input at higher rotational speeds, eventually dissipated as heat (MacNeil et al. 1985). Cooling is particularly important, and is usually achieved through a jacket around the cooling chamber, ball bearing and seal (Schiitte et al. 1983). Additional heat transfer area can be provided in larger mills using cooled end plate. It is also possible to cool the agitator and shaft (Rehficek and Schaefer. 1977, Schitte et al. 1986).

Bead mills are ideally suited to yeasts, which have a large diameter and are easily impacted by colliding beads. Bead milling has also been employed to disrupt the bacteria Brevibacterium ammoniagenes, Bacillus sphaericus, Escherichia coli and Lactobacillus confusus by Schitte et al. in a Netzsch LME 20 mill (1988). As expected, disruption efficiency for these smaller microorganisms is considerably less than that of yeast. Multiple passes were required in each case compared with single pass operation for yeast. Also the milling time was more in the case of Escherichia coli than yeasts. A very strong dependence of Methanomonas disruption on bead size was also apparent; with a significant drop in efficiency as bead size was increased. Bead mills have also been employed to disrupt the bacteria Arthrobacter (Bunge et al. 1992) and Bacillus subtilis (Rehficek J. 1971), the fungal organisms Aspergillus niger (Zetelaki K. 1969) and Neurospora sitophila (Baldwin and Robinson. 1994), and the algae Scenedesmus

3.10.1. Important operational parameters of bead-milling

Bead-milling is characterized by a number of parameters. The most important ones are listed below:

| Table. 3.10.1: Important operational parameters of bead milling process |
|---|---|
| Agitator speed | Feed rate of the cell slurry |
| Cell density | Size of the beads |
| Weight of the beads | Packing density of beads |
| Temperature | |

3.10.2. Agitator speed

The agitator is responsible for the energy input and the activation of the grinding elements in the grinding chamber. By increasing the impeller tip speed, the shear forces generated increase, as does the collision frequency. At the same time, the temperature increases depending on the packing density of beads in the grinding chamber. The erosion of the glass beads increases and also the necessary energy to drive the agitator. As reported in literature, there is no linear relation between the impeller tip speed and degree of cell disintegration, which can be attributed to changes in residence time distribution with variation of agitation speed at constant feed rate (Asenjo and Dunnil. 1981) and higher agitation speeds may lead to additional heating of the cell homogenate.

3.10.3. Effect of feed rate on microorganism disintegration

Theoretically, the degree of cell disintegration achieved per passage should approximately be inversely proportional to feed rate. An increase of one order of magnitude in the feed rate from 10-100 Lh^{-1} results in only 18 % decrease in the degree of disintegration of S. cerevisiae. On the other hand, release of intracellular enzymes from Brevibacterium ammoniagenes decreases more markedly with increasing feed rate. Considering this and the energy demand, it is advisable to operate high-speed agitator bead mills at high volumetric flow and utilize repeated passages, or in continuous large scale processes a two- or three-in-series configuration of mills in order to reach the necessary the necessary levels of cell disintegration.
3.10.4. Size of the beads

For the disruption of microbial cells, special lead free glass beads are required with diameter in the range of 0.2 – 1.5 mm. Glass beads of smaller diameter are better as by increasing the agitation speed, the energy content of the glass bead can be increased to quite high values. For the same volume the number of packed glass beads increases and also the void space in between the beads reduce. However, efficiency will also be limited as the smaller beads tend to float on the surface. For continuous milling at large scale the size of grinding elements are restricted to ≥ 0.4 mm for easy separation. The higher mass of larger glass beads, are able to carry high kinetic energy and lead to larger difference in the velocity profile of the glass beads and increased shear forces. Experimental evidences suggest that the optimal size of the glass beads relative to the size of microorganisms; for yeast diameters of greater than 0.5 mm and for bacteria less than or equal to 0.5 mm is considered optimal. Enzymes located in the periplasmic space are better solubilized by larger beads rather than the smaller ones (Baldwin and Robinson. 1990).

3.10.5. Bead loading

The amount of beads present in the grinding chamber is expressed usually in terms of percentage volume to the free volume of the grinding chamber. The packing density should allow an equal distribution of the beads without severe interference in the establishment of velocity profile. The optimal performance depends on the size of the glass beads employed. Working with 0.5 mm glass beads, a bead loading of 85% and for 1 mm glass beads 80% bead loading has been recommended (Augenstein et al. 1974). At a bead loading below 80% efficiency of cell disintegration is impaired and above 90%, heating becomes a problem and can cause denaturation of the desired biological molecule.

3.10.6. Specific weight of the grinding elements

The specific weight of the glass beads utilized for the disintegration of microbial cells is in the order of 2.7 ± 0.2 g/cm³. Alternatively ceramic beads made from partially zirconium oxide with specific weight of approximately 3.8 g/cm³ and zirconium oxide beads with specific weight of 5.4 g/cm³ can be used. The price of the grinding element rises sharply with increasing specific weight, but contrary to expectation, the cell disintegration doesn’t improve. Beads with high specific weight are successfully used
for wet milling with highly viscous products. However, there seems no advantage in handling suspensions with low viscosity (Schutte and Kula, 1983). For the glass beads, besides the price, the availability in different diameters and in rather narrow size distribution is a distinct advantage for processing different microorganisms.

### 3.10.7. Concentration of cell suspension

The cell concentration is not of much significance and doesn’t affect the effectively of the cell disintegration process. Morgan et al (1974) reported an identical degree of cell disintegration at lower cell concentrations of 4-20 % dry cell weight, while disrupting yeast cells, in bead mill. Similar observation was reported by Limon – Lason (1979) where it was reported that the disintegration was not much affected at lower cell concentration below 30% w/v (wet cell weight). Nevertheless, cell concentration is an important factor for industrial scale production, where economics and maximizing product concentration are the major challenges.

### 3.10.8. Temperature

A variation in temperature is only possible within a narrow range in order to avoid denaturation or deactivation of the solubilized proteins. Currie et al (1972) investigated the temperature as a parameter for the first order rate constant, disrupting baker’s yeast in a vertical Netzsch Moulinex KE5 mill and observed 20 % increase in disruption at 40 °C as compared to 4 °C. The temperature is difficult to precisely control, since a large portion of the energy introduced by the agitator into the homogenate is transformed into heat. This heat is subsequently removed by cooling system by passing chilled water through the jacket and the seal.

### Purification of recombinant proteins

A wide variety of protein purification techniques are available today, however different types of chromatography have been used in different recovery and purification processes due to their cost, efficiency and resolving power. The protein separation depends on various factors such as biological and physico-chemical properties: molecular size, net charge on the protein, bio-specific characteristics, hydrophobicity, etc.
3.11. Ion-exchange chromatography

This mode of chromatography exploits the charge density and extent of ionization of proteins. The separation is based on the ionic interactions between the protein and the chromatography matrix basically the binding of protein to a charged matrix. Proteins are polymers of amino acids which confer charges, positive / negative or sometimes none. Depending on the composition of and distribution of amino acids in a protein, it can exist as a positively charged or negatively charged molecule. This property is exploited when ion-exchange chromatography is performed. The amino acids contain positive charged moieties as well as negatively charged ones too. These groups ionize to different extents depending on the pH of the solution they are residing in. The net charge on a protein is dependent on the pKa and on the pH of the solution in accordance with Handerson – Hasselbalch equation.

\[
pH = pK_a + \log \left( \frac{\text{Ionised Form}}{\text{Unionised Form}} \right)
\]

For weak acid \hspace{2cm} Eq. a.3

\[
pH = pK_a + \log \left( \frac{\text{Unionised Form}}{\text{Ionised form}} \right)
\]

For weak base \hspace{2cm} Eq. a.4

Protein separations using ion-exchange chromatography are carried out mainly in columns packed with ion-exchangers. Depending on the charge of the exchanger, it can be cation exchanger (negatively charged) or an anion exchanger (positively charged). They are also termed as acidic or basic anion exchangers as the negative charge generates by the ionization of acidic groups and positive charge due to the ionization of basic groups.

3.11.1. Mechanism of ion-exchange chromatography

The ion-exchange mechanism is thought to be composed of five distinct steps:

1. **Diffusion of the ion to the exchanger surface.** This is the first step where the ion-exchanger is brought to starting stage, in terms of pH and ionic strength which paves way for the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter ions (usually simple anions or cations).

2. **Diffusion of the ion through the matrix structure to the exchange site.** After application of the sample and adsorption, in which solute molecules carrying the
appropriate charge reach the counter-ion on the gel. This is the limiting step in ion – exchange chromatography.

3. **Exchange of ions at the exchange site.** This is thought to be an instantaneous process and also attains equilibrium in the process. The counter ions are displaced from the matrix and the solute molecules bind reversibly to the matrix.

4. **Desorption and diffusion of the exchanged molecule or ion from the matrix and its pores.** Desorption is achieved by changing the salt concentration gradient and the solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first.

5. **The removal of substances or contaminants.** The column is passed with 1-2 M NaCl solution to remove those bound protein molecules, which are not eluted from the column under the previous experimental conditions. The column is then washed with distilled water / recalibration buffer for the next purification cycle.

Separation is obtained since different proteins have different degrees of interaction with the ion-exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often considerable, and since ion exchange chromatography is capable of separating species with very minor differences in properties. In addition to the ionic interactions, other types of binding may occur. These include Van-der Waals forces and non-polar interactions. Ion exchange separations may be carried out in a packed-bed column or expanded bed mode.

**3.11.2. Factors affecting ion – exchange chromatography**

A number of factors affect the binding, elution and separation of proteins in ion – exchange chromatography. The major ones are:

**3.11.2.1. The stationary phase**

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. It is possible to have both positively and negatively charged exchangers. Positively charged exchangers have negatively charged counter-ions...
(anions) available for exchange and are called anion exchangers, the functional groups mainly include sulphonates (-SO$_3^-$), Carboxylate (-COO$^-$), etc. Negatively charged exchangers have positively charged counter-ions (cations) and are termed cation exchangers e.g. quaternary amines (-N$^+$R$_3$), Diethyl ammonium (-NH$^+$(CH$_2$CH$_3$)$_2$), etc. The matrix may be based on inorganic compounds, synthetic resins or polysaccharides. The characteristics of the matrix determine its chromatographic properties such as efficiency, capacity as well as its chemical stability, mechanical strength and flow properties. The nature of the matrix will also affect its behavior towards biological substances and the maintenance of biological activity. Ion exchangers based on dextran (Sephadex), followed by those based on agarose (Sepharose CL-6B) and cross-linked cellulose (DEAE Sephacel) were the first ion exchange matrices to combine a spherical form with high porosity, leading to improved flow properties and high capacities for macromolecules. Subsequently, developments in gel technology have enabled this macro-porosity to be extended to the highly cross-linked agarose based media such as Sepharose High Performance, Sepharose Fast Flow and Sepharose Big Beads, etc. These materials provide strength to the matrix and prevent them from being distorted or deformed during the chromatography run, where sometimes cross column pressure increases. Non-porous polymer matrices, e.g. Mini-Beads, are used for extremely high resolution micro-preparative or analytical separations. All exchangers are characterized by total exchange capacity, which is defined as the number of milli-equivalents of exchangeable ions available, either per gram of dry resin or per unit volume of hydrated resin. Sometimes the capacity is also expressed in terms of amount of an arbitrary protein bound per unit volume of the resin in the hydrated state e.g. lysozyme, BSA etc. This gives an indication of the degree of substitution and help in deciding the scale of a particular purification process.

3.11.2.2. The mobile phase

The choice of the mobile phase depends mainly on the nature of protein, e.g. stability, degree of ionization, molecular mass, iso-electric point or any other specific requirement. The buffer, in which the protein is most stable, should be used in chromatography especially at large scale purifications. The binding depends on the charged state of proteins that are weakly ionized. Hence the loading or the capture buffer used should be of enough ionic strength to allow the binding of the protein to the matrix and maintain its pH when the protein is added to it.
3.11.2.3. Effect of pH and ionic concentration

Many biological molecules and compounds, especially proteins are stable in between a fairly narrow pH range so the exchanger selected must operate within this range along with the pH range of the buffer used. If the protein to be purified exists as positively charged, cation exchangers should be used (and vice versa) also, the pH of the buffer should be in the pH range, where the protein is maximally stable. In general, cationic buffers such as Tris, pyridine, alkylamines are used in conjugation with anion exchangers and anionic buffers such as acetate, barbiturate and phosphate are used with cation exchangers. The precise initial buffer pH and ionic strength should be just enough to allow the binding of the sample components to the exchanger matrix. Equally, a buffer of the lowest ionic strength should be used for the subsequent elution of the components.

3.11.2.4. Effect of binding capacity of exchanger

The binding capacity of the column exhibits a very precise balance between the yield and quality of the protein in the eluted fractions. The amount of the sample that can be applied to the column is dependent upon the size of the column and the capacity of the exchanger; it is normally the product of the column volume and the binding capacity of the exchanger per unit volume. The conditions are chosen in such a way that the entire sample loaded gets bound at the top of the column. In this case, large volume of diluted sample can be applied which effectively introduces a concentrated stage. The exchanger is made up of polymers cross-linked to provide strength and platform for separation. The porous nature of exchanger beads provide larger surface area for smaller proteins that can move into the pores to bind the immobilized ligands inside, thereby increasing the capacity of the exchanger per unit volume, while larger proteins are restricted to the surface and result in lower amount of binding. If the binding capacity of the exchanger is too high or the binding of the solute molecule may be very strong and it’s desorption may need the application of buffers that might denature them. However, lower binding capacities are not preferred for large scale and industrial applications.

3.12. Hydrophobic interaction chromatography

Hydrophobic interactions have a great importance in the biological systems. They are the dominant force in protein folding and structure stabilization (Privalov and Gill. 1988; Dill K A. 1990; Murphy et al. 1990; Makhatafaze and Privalov. 1995) and play
an important role in other biological processes like antibody–antigen reactions (Dandliker et al. 1967; Van Oss et al. 1986), enzyme–substrate reactions and the maintenance of biological membranes (Tanford. 1973). These processes are driven by hydrophobic effect and according to thermodynamic analysis of calorimetric data, these are accompanied by significant entropy and heat capacity changes at room temperature (Vailaya and Hovath. 1996).

Proteins are polymers of amino acids. Amino acids can be classified into hydrophilic and hydrophobic based on their nature. The number of hydrophobic amino acids, their distribution and hydrophobicity result in the characteristic nature of each protein. Hence each protein interacts with different affinities towards hydrophobic supports or matrices. This variation in the hydrophobicity of proteins provides another means by which these biomolecules could be fractionated using hydrophobic interaction chromatography (Kennedy R M. 1990; Ochoa. 1978; Vogel et al. 1983; Lindahl and Vogel. 1984).

3.12.1. Mechanism of hydrophobic interaction chromatography

Tiselius in 1948 was the first to demonstrate that amino acids and proteins bound to neutral supports in high concentration of alkaline phosphates. The name hydrophobic interaction chromatography was introduced by Hjerten in 1973 to describe salt mediated separation of proteins on weakly hydrophobic carbohydrate gel matrices. The adsorption of proteins on the matrix increases with increase in salt concentration in the mobile phase and the elution is achieved by decreasing salt concentration of the eluent (Melander and Hováth. 1977; Fausnaugh and Regnier. 1986; Roe S. 1989). Hence, the term salt promoted chromatography could be used for this type of chromatography (Porath J. 1986). It was also proposed as salt promoted adsorption or salt prompted adsorption chromatography (SPAC) as an alternative expression to HIC and other type of solute–adsorbent interactions that take place in the presence of high concentrations of neutral salt (Porath et al. 1973). Further, it was regrouped in various classes (Porath J. 1990; Berna et al. 1998) but HIC is still the most popular and established. The influence of different salts on hydrophobic interactions follows the Hofmeister (lyotropic) series for the precipitation of proteins from aqueous solutions (Pahlman et al. 1977; Roe S. 1989).

*Increasing salting-out effect*

\[ \text{Anion: } PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^- \]
The salts at the beginning of the series promote hydrophobic interactions and protein precipitation (salting out effect) and are called anti-chaotropic. These are called to be water structuring, whereas the salts at the end of the series randomize the structure of the liquid water (salting-in or chaotropic ions) and thus tend to decrease the strength of hydrophobic interactions (Porath J. 1987). The retention mechanism of proteins on HIC has been widely studied but none of the proposed theories have enjoyed general acceptance.

3.12.2. Factors affecting hydrophobic interaction chromatography

To obtain success in a chromatographic process two major elements have to be considered, a stationary phase and a fluid mobile phase.

3.12.2.1. The stationary phase

The various kinds of stationary phases could differ on the type of matrix or support. The most widely used ligands for HIC are linear chain alkanes with or without a terminal amino group. Phenyl (or other aromatic groups) is also used as a ligand with good results due to mixed hydrophobic and aromatic (π–π) interactions. At a constant degree of substitution on the matrix the n-alkane ligands constitute a homologous series in a hydrophobicity scale (Tanford C. 1972):

Methyl < Ethyl < Propyl < Butyl < Pentyl < Hexyl < Heptyl < Octyl

The hydrophobicity and the strength of interaction increases with the increase in n-alkyl chain but the adsorption and selectivity may decrease. An increase in the degree of substitution of immobilized ligand leads to an increase in protein binding capacities of HIC gels, due to higher probability of forming multi point attachment between the protein and the ligand (Hjertén et al. 1974). The relation between the protein retention and ligand density apparently depends on the size of the protein, in spite of that, the protein surface hydrophobicity should be considered to be the most influential factor (Fausnaugh et al. 1984).
3.12.2.2. The mobile phase

The protein retention in HIC not only depends on the stationary phase but also on the characteristics of the mobile phase, such as the type and concentration of the salt, pH, temperature and additives. The effect of salt composition on the protein retention follows the order of the salts in the lyotropic series for precipitation of proteins or for their positive influence in increasing the molal surface tension of water (Melander and Horváth. 1977; Melander et al. 1984). Salts such as sodium, potassium or ammonium sulphates are the most effective in promoting ligand protein interaction. However, magnesium sulphate and magnesium chloride do not enhance the protein retention despite the fact that they increase the surface tension of water. It appears to include other complex interactions, apart from having the effect on the surface tension of water, such as specific interaction between the salt and the protein molecules, which may not change the protein structure or the hydration sphere (Arakawa and Timasheff. 1984; Fausnaugh and Regnier. 1986). Also the concentration of salt strongly influences the selectivity of protein adsorption and this influence is different and dependent both on the stationary phase and the buffer salts (Osacarsson and Karsnas. 1998). The amount of bound protein increases with increase in the ionic concentration or the amount of salt in the loading buffer. The adsorbed proteins are eluted by gradient or stepwise elution at decreasing salt concentration in the eluent. The salts used in HIC should be highly soluble to avoid precipitation, when they are added in high concentration in the eluent to drive the hydrophobic interaction.

3.12.2.3. Effect of pH on hydrophobic interaction chromatography (HIC)

The pH of the mobile phase also affects the hydrophobic interactions between protein and ligand molecules and its a critical factor in HIC. pH can directly affect protein retention in hydrophobic interaction chromatography (Hofstee B H J. 1973; Strop et al. 1983; Sanz et al. 1998). Usually, an increase in pH value (up to 9 – 10) decreases the hydrophobic interactions between the protein and the hydrophobic ligands, due to increased hydrophilicity prompted by the change in the charge of the protein (Hjerten et al. 1973; Porath J. 1986). On the other hand a decrease in pH results in an apparent increase in the hydrophobic interactions but this phenomenon is highly dependent on the nature of the protein under study.
3.12.2.4. Effect of temperature on hydrophobic interaction chromatography

Temperature is yet another factor which has a significant effect on the retention of proteins in HIC. With the increase in temperature, the retention of the protein increases and lowering the temperature generally promotes the protein elution (Hjerten et al. 1974). However an opposite protein retention behavior can occur sometimes. When the temperature affects the conformational state of protein and their solubility in aqueous solution, retention in the HIC column may decrease. Even the role of temperature is not so simple, this parameter can be used to achieve weaker interactions and promote elution in separation of proteins under mild conditions, without denaturation (El Rassi Z. 1996).

3.12.2.5. Effect of Additives on hydrophobic interaction chromatography

Certain additives can be used in HIC, not only to improve protein solubilization or to modify protein conformation, but also to promote the elution of the bound proteins. The most widely used additives are water miscible alcohols, e.g. ethanol and ethylene glycol, detergents e.g. Triton X-100 and aqueous solutions of chaotropic salts. The non-polar part of alcohols and detergents achieve the displacement of bound proteins due to the competition for the ligands on HIC stationary phase. When ionic detergents are bound to HIC media, the separation mode is a mixed ion-exchange hydrophobic interaction process, due to the presence of charged group in the detergent (Janson and Ryden. 1993). Chaotropic salts promote desorption of the proteins by affecting the ordered structure of water and / or of the bound proteins but tend to denature or inactivate proteins at high concentrations. Hence, their use in elution is usually considered a last resort, when milder conditions do not promote protein recovery. The major drawback using additives in a preparatory chromatography process is their removal after the recovery of protein which again requires investment, labor and time affecting the overall yield and process economics

3.13. Expanded Bed Chromatography

A majority of proteins produced by cultivation of microorganisms, animal and plant cells involve suspended particles and a combination of many biomolecules having different physico-chemical properties. The suspended particles include whole cells along with their fragments. When the desired substance is present within the cells, a cell
A breakage step is necessary to release the intracellular product. Isolation of the product of choice usually involves the following steps:

1. Separation of cells from the surrounding fluid i.e. clarification
2. Isolation of a rather impure product from the fluid i.e. concentration
3. Purification of the impure product

Depending on the extent of the separation required (determined by the nature of step 2), the first step may take the form of screening, dead-end filtration, cross flow filtration, settling and decantation or centrifugation, or a combination of two or more of these operations. Sometimes coagulation and/or filter aids may be used to assist the processing. If such additives are to be avoided, filtration is the best way out. The standard techniques used for the removal of cells and cell debris are centrifugation and microfiltration. The centrifuges in current use are well suited to industrial processes, however, the cost and effectiveness of centrifugation is highly dependent on particle size, density difference between the particle and surrounding liquid and viscosity of the feed stock. Small cells (E. coli, or cell homogenates), small particle size and high viscosity reduce the feed capacity during centrifugation and sometimes make it difficult to obtain a completely particle-free liquid in which the clearance value of the particles should be in excess of 99 to 99.9% (Datar and Rosen. 1987). It has also been shown that centrifugation is not efficient enough to clarify cell culture broth for direct application onto chromatographic column (Berthold

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Fig. 3.13.a. Process pathways with conventional downstream process and after inclusion of expanded bed chromatography
To obtain particle-free broth that can be further purified by traditional packed bed chromatography (PBC), centrifugation is usually combined with cross flow microfiltration (Bailey et al. 1990; Fane and Radovich, 1990). A major advantage of cross flow filtration is the relative ease with which the process can be operated as a contained system. Though microfiltration yields cell free broth, the flux of liquid per unit membrane area often dramatically decreases during the filtration (when the solid content approaches 30%) (Tutunjian R S. 1982; Schutte H. 1982). Problems arise from fouling of the microfiltration membrane and form concentration polarisation, both of which may have adverse effect on the process. The combined use of centrifugation and filtration often results in long process time, use of comparatively large units causing significant expenditure on process and equipment maintenance. Disruption of cells also gives rise to problems in microfiltration; the disruption of the cell wall generates many small particles including those released from within the cells. The matter is complicated further by the release from the cells of nucleic acids which may cause a significant increase in viscosity. One of the keys to reduce the costs of a biotechnological production process is the simplification of the downstream process (Spalding B J. 1991). This may be achieved by the reduction in the number of steps involved in the process. Integrated technologies have aimed at combining single steps of a protocol into new unit operations. Standard packed bed chromatography requires particulate material be removed from the feed, since the packed bed acts as a dead-end filter and is easily blocked by the particulates. An alternative to this is the adsorption on to a resin in a stirred tank with feed stocks. At the end of the adsorption phase, adsorbent particles are separated from the depleted feedstock using a filtration or sedimentation system that is able to distinguish between adsorbent particles in the feed. Washing and elution of the loaded adsorbents may then be carried out either in batch or packed bed mode. This method has, for instance, been used for many years on a commercial scale for the isolation of plasma coagulation factor IX using DEAE Sephadex (Brummelhuis, 1980). The well-mixed batch adsorption is a single-stage process that requires more adsorbent to achieve the same degree of adsorption as compared to packed bed chromatography. Other disadvantage of this process is the mechanical difficulty in handling the adsorbents. Another approach to increasing the efficiency of batch adsorption is the use of submicron ion-exchange particles, which are later removed from the cell homogenate by centrifugation (Vorauer-Uhl et al. 1993).
Expanded bed chromatography can be defined as the mode of chromatography where the matrix is stably fluidized by the upward flow (against the gravity) of sample/buffers. This results in larger void spaces in between the beads, and allows particle-containing feed-stocks to be easily applied without clogging or fouling of the column.

3.13.1. Adsorption on expanded bed

Chase H A (1994) and Draeger and Chase (1990; 1991; 1992) introduced the concept of adsorption of proteins on expanded beds. They documented that the bed voidage is larger than that in packed/sedimented beds. Expanded beds are sometimes considered as an integral part of the fluidised bed technology; some authors do not differentiate between the two expressions (Hjorth R. 1997).

3.13.2. Principle of expanded bed chromatography

Expanded beds are based on fluidisation. When a liquid is pumped at a low velocity to the bottom of a sedimented bed the liquid flows through the interstitial space of the sedimented bed, without causing adsorbents to move. With increase in the superficial velocity, the pressure drop in the liquid through the bed increases linearly. When the pressure drop equals the force of gravity on the particles and the adsorbents begin to move. First the bed expands slightly with the adsorbents still in contact. As the porosity increases, the rise in pressure drop is more gradual than before. At this time, the bed is in the loosest possible condition with the adsorbents still in contact. As the velocity is increased further, the adsorbents separate out and true fluidisation begins. The pressure drop sometimes diminishes marginally. On increasing the onward flow rate, the particles move more and more vigorously, swirling about and travelling randomly in all directions resembling a boiling liquid. Linear velocity of fluid between the particles is much higher than the velocity in the space above the bed. Consequently nearly all the particles drop out of the fluid above the bed. On increasing fluid velocity further, the voidage (porosity) of the bed rises; the bed of solids expands, and its density falls. The pressure drop rises with the rise in fluid velocity but gradually than when the solid particles were stationary. The adsorbent particles have high density, but with a distribution in size. When there is constant upward flow, the beads are suspended and each particle finds its own equilibrium position; the bed is expanded and stable. This is called the relaxation point and the velocity required to cause relaxation of solid particles is called minimum fluidisation velocity, which characterizes transition between the
sedimented and expanded state. At the relaxation point, column back pressure is in equilibrium with the force due to the weight of the adsorbent particles. Further increasing the flow-rate merely causes expansion of the particle bed and a constant back pressure in the expanded bed.

### 3.13.3. Operation

Loading the adsorbents in a column generates a random distribution of small and large particles in the sedimented bed. During equilibration, when the bed is expanded the beads get entrained in the order of their size and find their own equilibration position. Expanding the bed by a factor of 2 or 3, increases the voids between the adsorbent particles and the bed porosity. After expanding the bed stably, the feed containing cells, cell debris or other particulate matter is applied. As the physical properties of the feedstock are different from those of the buffer used for equilibrations, especially the viscosity being high, the bed expands further if the flow rate is not decreased. An intensive washing step is necessary to remove particulates and weakly adsorbed proteins as particulates may get entrapped in the column. After washing, the flow is stopped, the adsorbent allowed to settle and finally the upper adapter is lowered. If necessary, further washing can be done in the packed mode in either flow direction. Elution is generally carried out in the packed configuration as smaller volume is needed to elute the product (Fig. 3.13.3.a). Due to reasons of biological safety, movable parts may be undesirable and a closed system may be preferred. Therefore, the upper adapter can be fixed and

![Fig. 3.13.3.b. Steps involved in a typical expanded bed chromatography process](image-url)
washing and elution carried out in the expanded mode, but it results in increase in elution time and volume (McCormick D K. 1993).

3.13.4. The Column

The column has a significant impact on the formation of stable expanded beds. Streamline (Amersham Pharmacia Biotech, Uppasala, Sweden) columns are equipped with a specially designed liquid distribution system to allow the formation of a stable expanded bed as the pressure drop over an expanded bed less. The distributor plate in an expanded bed column is designed to produce a plug flow by itself. Consequently, it is necessary to build in an additional pressure drop into the distribution system. Besides generating a pressure drop, the distributor has to direct the flow in the vertical direction alone. Any radial flow inside the bed will propagate turbulence through the column and make the bed unstable. The distributor system prevents the adsorbent from leaving the column by a net mounted on the side of the distributor, facing towards the adsorbent. The net must have a mesh size that allows particulate materials to pass while retaining the adsorbents in the column. Streamline columns are also equipped with a movable adapter for adjusting column height during the different stages of an expanded bed adsorption cycle.

3.13.5. The matrix

Tailoring the chromatographic characteristics of an adsorbent for use in expanded bed includes careful control of the sedimentation velocity of the adsorbent beads. The sedimentation velocity is proportional to the density difference between the adsorbent and the surrounding fluid multiplied by the square of the adsorbent particle diameter. To achieve the high throughput required in industrial applications of adsorption chromatography, flow velocity must be high through the complete purification cycle. The first result reported from expanded bed adsorption using conventional chromatographic adsorbents based on agarose revealed an obvious need for particles with higher sedimentation velocity to allow the operation of expanded beds at high flow velocities without the beads being carried out of the column by the lifting liquid flow (Draeger and Chase, 1990).

Streamline adsorbents (GE lifesciences) are based on 6% crosslinked agarose with quartz particle inside the core, a material proven to work well for industrial scale chromatography, as claimed by the company (Carlsson et al. 1995). The macroporous
structure of the highly cross-linked agarose matrices combines good binding capacities for large molecules, such as proteins, with high chemical and mechanical stability (Chase H A. 1994). High mechanical stability is essential for matrix used to reduce the attrition when particles move about freely in the expanded bed. It is resistant to high salt concentrations, stable at room temperature in buffers of pH range 4-9. The chemical and mechanical stability of agarose matrix gel can be increased by crosslinking with epichlorohydrin (Porath et al. 1971; Kristiansen, 1974), 2,3-dibromo propanol (Kristiansen T, 1974) or divinyl sulphone (Porath et al. 1975), especially in aqueous medium, in both acidic and alkaline regions (pH 3-14). Crosslinking also increases stability of matrix in organic solvents such as ethanol, dimethylformamide, tetrahydrofuran, acetone, chloroform, dimethyl sulfoxide, dichloromethane and dichloroethane and thermal stability up to 121 °C. The modified agarose matrix in Streamline adsorbents is more elastic (less brittle) compared to inorganic materials such as glass or ceramic. The mechanical stability of Streamline matrices have been verified by repeated expansions and sedimentations and by subjecting the adsorbent to different shear forces (Hansson K A. 1995).

3.13.6. Advantages and applications of expanded bed chromatography

The reported applications of stable expanded beds for the purification of proteins from crude feed or lysates are, at present are sparse. But considerable industrial interest has resulted in the development of unpublished, in-house procedures. Expanded Bed Chromatography has been successfully used for the capturing of target molecules from crude and unclarified feed material. The applications cover different types of feed material and illustrate a variety of separation techniques, including ion-exchange, affinity and hydrophobic interaction chromatography. Expanded bed anion exchange has been used for pilot scale recovery of recombinant human placental annexin-V from *E. coli* homogenate (Barnfield F A et al. 1994). It has been used to recover proteins from *E. coli* homogenates, without any prior centrifugation or microfiltration (Frej et al. 1994).

Adsorption of the target molecule to an adsorbent in expanded bed also eliminates the need for particulate removal (by centrifugation or microfiltration). Fluidized beds have been used in industry for many years for the recovery of antibiotics including batch processing technique for the recovery of streptomycin, (Barthels et al. 1958) and semi-continuous system for novobiocin (Belter et al. 1973). A method has also been
published describing the successful capture of immunomycin from *Streptomyces* culture at large scale (Galliot *et al.* 1990). Several attempts have been made to stabilize fluidized bed to accomplish a multi-stage fluidized bed reactor with separation characteristics similar to packed bed chromatography. Draeger and Chase were able to create a stable fluidized bed, with chromatographic characteristics similar to packed bed, by using conventional agarose column, designed with a liquid distribution inlet to give a plug flow in the column. The application of mixtures of proteins and cells onto these expanded beds promise the potential of the technique for recovery of proteins from a particle-containing feed-stock (Draeger and Chase. 1990; 1991; 1992).

3.14. Derivatization of proteins with polyethylene glycol

Poly-(ethylene glycol) or PEG is a neutral, hydrophilic polyether which exhibits little reactivity unless modified with functional groups. It is used as a non-toxic, non-immunogenic lubricant or carrier in pharmaceutical formulations. Covalent attachment of PEG-groups to proteins results in active conjugates that are non-immunogenic, non-antigenic and have greatly increased *in-vivo* circulation half-lives (Abuchowski *et al.* 1977). These changes appear to be mainly due to significantly increased molecular size (hydrodynamic radii), surface alteration and antigenic domain protection (masking effect) by PEG polymers (Harris J M. 1992; Harris and Zalipsky. 1997; Veronese and Harris. 2002; Zalipsky. 1995). PEGylation of therapeutic proteins reduces renal clearance rates and protects from proteolytic and other degradation, resulting in enhanced medical efficacy. Other benefits of PEGylation may include improved physical and thermal stability, as well as aqueous solubility. Specific chemistries can be used to target conjugation to a variety of sites on a protein (Zalipsky. 1995). The most common chemistry targets the ε-amino group of surface lysine residues, which typically account for approximately 10% of amino acids in a protein presenting both opportunities and challenges. Their availability makes conjugation straightforward but the large number of conjugation sites presents difficulty in obtaining a specific number of PEG adducts and gives rise to PEGamer mixtures. In one simple approach amino group modification can controlled to some extent by reaction pH so that conjugation tends to occur at the ε-amino of the N-terminal (Kinstler *et al.* 2002; 1996). Sulfhydryl group of free cysteine groups or carboxyl groups may also be targeted just like lysine residues. Proteins can be engineered by site-directed mutation or otherwise chemically modified to fabricate a suitable conjugation site (Chapman A P. 2002; Chapman *et al.*
1999; Roberts et al. 2002). Such approaches have disadvantages in terms of time to market, cost of development and risk compared with developing a PEGylated form of an already-approved protein.

### 3.14.1. PEGylation reaction

In the case of protein PEGylation, the high cost of raw materials (particularly highly purified native protein) implies that maximizing reaction extent and specificity are critical objectives. In general, only pure protein preparations are PEGylated. This means that PEGylated proteins, which require additional reaction, separation and analysis, are inherently more expensive to produce. Problems in validation and separation are the main reason for not PEGylating crude protein samples (Hoyle P C. 1991). Modern trends are to carry out PEGylations using PEGs functionalised with groups which tend to readily hydrolyse. Such groups, such as NHS or SPA require specific care in regard to storage and often require use of excess molar ratio's of PEGylating agent to protein.

One of the major disadvantages is that the reactants, products and by-products remain in contact throughout the reaction process and must be separated after completion of the reaction. Batch reaction is particularly suitable when site-specific PEGylation chemistry is used. When a less specific chemistry is employed, such as conjugation via ε-amino groups of lysine residues, both under- and over-PEGylated products are inevitable.

Some workers have addressed the issue by using packed-bed or “on-column” PEGylation in attempt to influence both the site and the extent of conjugation. A different approach to column PEGylation, size exclusion reaction chromatography (SERC), was used by Fee (2003), who exploited the differing linear velocities of species of differing sizes in SEC to control reaction extent. Single pulses of the protein and the activated PEG are injected into the column with lowest molecular size first. The larger reactant has a higher linear velocity so it catches up to the smaller one such that a moving reaction zone is formed as they migrate through the column. As the PEGylated protein formed is larger than either reactant, it moves ahead of the reaction zone. With a sufficient length of column, both reaction and separation of all species can be achieved in a single unit operation. The approach is particularly useful with regard to PEGylation as many native proteins of therapeutic interest are less than 20 kDa, and smaller in colloidal size than even a 5 kDa PEG molecules.