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REVIEW PAPER

High Yield Production of Heterologous Proteins with *Escherichia coli*

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ABSTRACT

The demand for therapeutic recombinant proteins is set to see a significant increase over the next few years. As a consequence, the processes used to produce these proteins must be able to meet market requirements. Recombinant proteins have gained enormous importance for clinical applications. The present paper reviews the fermentation process for high yield scalable production of heterologous recombinant proteins in *Escherichia coli*. Influence of process parameters for the standard fermentation processes are discussed, and alternative methods that solve the limitations are reviewed together with the methods that yielded in higher productivity of *E. coli* process. The common problems are scale-up issues, plasmid instability, acetate accumulation and substrate inhibition in the high cell density bioreactor production system. Methods to overcome these issues are described. Solving the problems makes ideal condition for high yield production with *E. coli* expression system as compared to other systems.

Keywords: Fermentation, *Escherichia coli*, scale up, media, yeast extract, high cell density

1. INTRODUCTION

Recent developments in biotechnology involve production of materials like vaccines, recombinant proteins, monoclonal antibodies, etc. Demand for these biological products has increased because they have been used for therapeutic and diagnostic purposes. The key component for the commercial success of any biopharmaceutical product is the ability to achieve large-scale manufacture. The ultimate goal of recombinant fermentation research is the cost-effective production of desired protein by maximising the volumetric productivity, i.e., to obtain highest amount of protein in given volume in least period of time. Such bio-processing for recombinant protein using genetically modified organism requires a stable high-yielding recombinant culture, a high productive fermentation process and cost-effective recovery and purification procedures. *Escherichia coli* has been most widely used as host for the expression of recombinant proteins[1-4].

Enormous data are available on advantages of using *E. coli* as expression system, its cell biology, fermentation process development and its ability to produce large quantities of recombinant proteins in an inexpensive way. In past 20 years, recombinant DNA technology has enabled to produce huge quantities of therapeutic proteins that might otherwise have been difficult to produce. Recombinant protein expression using *E. coli* as host is frequently associated with the formation of intracellular aggregates as inclusion body[5]. The volumetric yield of the protein is thus a function of both unit cell concentration and specific cellular protein yield. Optimisation of high cell density fed-batch fermentation process is thus one of the key steps for enhancing the volumetric yield of recombinant proteins[6-7].

High cell density fermentation is a major bio-process engineering consideration for enhancing the overall yield of recombinant proteins in *E. coli*. Understanding of basic biological aspect of the expression system at molecular level and translating this information at process level is imperative for efficient and cost-effective production of therapeutic compound. While maintaining a stable plasmid of interest, parameters that influence the high cell density fed-batch aerobic growth of *E. coli* have been analysed[8]. Novel ways of fed-batch fermentation process considering most of these factors have been discussed in detail to maximise the volumetric yield of recombinant proteins expressed as inclusion body in *E. coli*. The selection of a particular expression system requires a cost breakdown in terms of process, design, and other economic considerations. The advantages of *E. coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins[9-10]. Fed-batch processes have most often been used to obtain high cell density[11-15]. Cell concentrations of about ~100 g dry cell weight per litre can be yielded by fed-batch culture of both non-recombinant and recombinant *E. coli*[16-18]. However, this technique has several drawbacks, including substrate inhibition, limited oxygen transfer capacity, the formation of growth inhibitory by-products, and limited heat dissipation.

Several feeding strategies have been used for increasing cell concentrations in recombinant cultures, such as constant feed, step-wise increase in feed, feeding based on a feedback control technique so as to keep the dissolved oxygen or p*H* constant and exponential feed[18,19-23]. Exponential feeding is widely used so that the cells can be grown at the desired

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specific growth rate, preventing the formation of toxic by-products like acetate[24-27]. The feeding method to maintain the desired specific growth rate is fairly simple and amenable to feed-forward control using substrate balance equations[28].

Recombinant *E. coli* can be grown to high densities in common media such as Luria broth (LB) [29-31], synthetic M9 minimal medium[32], Terrific broth (TB) [32-34] and Super broth (SB) [35-37]. The bacterium is generally grown under aerobic conditions because anaerobic growth provides less energy for metabolic processes such as protein synthesis[38]. To ensure that oxygen supply does not become limiting, the fed-batch operation is used extensively in cultures[20,25.39]. This improves biomass and recombinant protein yield relative to batch culture. Fed-batch operation overcomes possible limitations due to a high concentration of substrate and enables growth to be prolonged in comparison with traditional batch fermentations. A major objective of fermentation processes is to maximise the volumetric productivity. High cell density cultures are the first step to achieve this goal. The objective of the present review is to emphasise on the importance of high productive fermentation of recombinant proteini in *E. coli*. General scheme for large-scale fermentation process is shown in Fig. 1. Some commonly used therapeutic products and their manufacturers are shown in Tables 1 and 2.

2. *Escherichia Coli* EXPRESSION SYSTEMS

In the industry, the selection of the host cell will be strongly influenced by the type and use of the product, as well as economic or intellectual property issues. A number of new, sophisticated host-vector combinations have become available in recent years. However, their use in commercial applications lags behind. The T7-based pET (Novagen) expression system[38] with more than 40 different pET plasmids available commercially is, by, far the most used in recombinant protein preparation.Systems using the λPL promoter/cI repressor (e.g., Invitrogen pLEX), Trc, Tac promoter (e.g., GE pTrc, pGEX), ara*BAD* promoter (e.g., Invitrogen pBAD) and hybrid lac/T5 (e.g., Qiagen pQE) promoters are common. *E. coli* K12 derivatives are most widely used, often with protease-deficiencies and other mutations. Recently, B strains, e.g. BL21, gained in popularity due to their efficiency in recombinant protein expression[3,4]. Further developments make the use of *E. coli* even more attractive due to product secretion (e.g., recombinant peptide) to the extracellular space[39].

Different *E. coli* B and K12 strains are the main work horses for expressing non-glycosylated peptides and proteins at research level and in the industry. *E. coli* is genetically well characterised and efficient expression of recombinant product to more than 50 percent of total cell mass has been reported[40]. The selection and design of the expression plasmids influence synthesis rates, plasmid copy number, the segregational plasmid stability and therefore productivity and regulatory issues. For industrial applications, selective pressure by antibiotics is mainly maintained in pre-cultures; main cultures are usually grown without selective pressure.

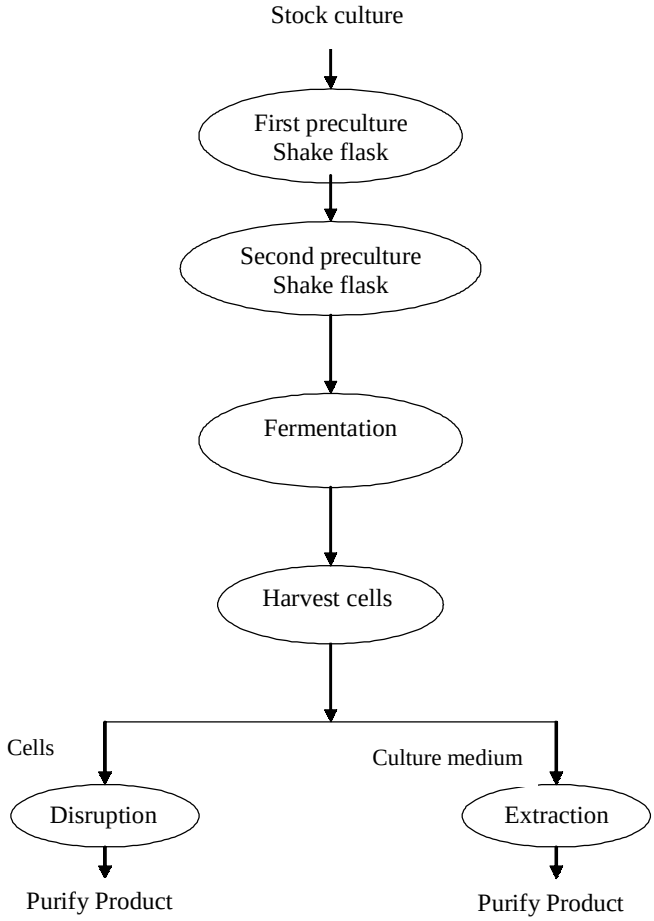

Figure 1. General scheme for large-scale fermentation process.

Table 1. List of some therapeutics and their manufactures in India

Product	Brand name	Company
Insulin	Wosulin	Wockhardt
Insulin	Insugen	Biocon
Erythroprotein	Hemax	Hindustan Antibiotics
Erythroprotein	Wepox	Wockhardt
Erythroprotein	Ceriton	Ranbaxy
Erythroprotein	Shanprotein	Shantha Biotechnics
Hepatitis B vaccine	Shanvac B	Shantha Biotechnics
Hepatitis B vaccine	Revac B	Bharat Biotech
Hepatitis B vaccine	Enivac HP	Panacea Biotech
Hepatitis B vaccine	Biovac	Wockhardt
Hepatitis B vaccine	Gene Vac-B	Serum Institute
Hepatitis B vaccine	Bevac	Biological E
Streptokinase	Indikinase	Bharat Biotech
Streptokinase	Shankinase	Shantha Biotechnics
Streptokinase	STPase	Cadila Pharmaceuticals
Interferon α-2b	Shanferon	Shantha Biotechnics

Table 2. List of some therapeutics with their heterologous expression host *E. Coli* and their manufactures abroad

Product	Company
Asparaginase	Merck
r Cholera toxin B subunit	SBL Vaccine
rh B-type natriuretic peptide	Scios/Johnson & Johnson
Tissue plasminogen activator	Roche
rh Insulin	Eli Lilly, Aventis
rh Growth hormone	Genentech, Eli Lilly
h Parathyroid hormone	Eli Lilly
rh Interferon α-2a and 2b	Hoffmann-LaRoche, Schering
Interferon alfacon-1	Valeant
r IL-2-diphtheria toxin fusion	Seragen / Ligand
rh IL-1 receptor antagonist	Amgen
r IL-2	Chiron
r Interferon β -1b	Schering AG, Chiron
r Interferon γ-1b	Genentec

The ideal expression vector combines medium-to-high copy numbers with tight regulation of gene expression to achieve rapid cell growth to high densities before the induction phase[8,41].

3. FERMENTATION

Protein production in *E. coli* can be increased significantly using fermentor culture systems, which can be classified into three groups: batch, fed-batch, and continuous[1,42-43]. These methods can achieve cell concentrations about 100 g/l of dry cell weight and can provide cost-effective production of recombinant proteins. The development and design of fermentation process and fermenter itself play a key role for achieving productivity and robustness at scale up[44].

3.1. High Cell Density Fermentation

High cell density culture (HCDC) techniques for culturing *E. coil* have been developed to improve productivity, and also to provide advantages such as reduced culture volume, enhanced downstream processing, reduced wastewater, lower production costs and reduced investment in equipment. High cell density culture systems also suffer from several drawbacks, including limited availability of dissolved oxygen, carbon dioxide levels which can decrease growth rates and stimulate acetate formation, reduction in the mixing efficiency of the fermentor, and heat generation[16-17,40,44]. A major challenge in the production of recombinant protein at high cell density is the accumulation of acetate, a lipophilic agent that is detrimental to cell growth. A number of strategies have been developed to reduce acetate formation, but these suffer from several drawbacks. This problem was resolved through the metabolic engineering of *E. coli*[45]. The reduction in acetate accumulation caused a significant improvement in the production of recombinant protein[46]. Mutant strains of *E. coli* that are deficient in other enzymes have also been developed and shown to produce less acetate and higher levels of recombinant proteins[47].

In high cell density fermentation, maximising cell concentration helps in increasing the volumetric productivity of recombinant proteins. For example, optical density of about 124 resulted for high cell density culture for production of recombinant ovine growth harmone in *E. coli*[40]. It is also essential that cell growth be achieved in optimal time period to improve the overall production of the recombinant protein. Toxicity of acetate, slow growth rate, instability of plasmid, depletion of amino acid pools to sustain high rate of protein synthesis affect the specific cellular yield of recombinant protein at high cell concentration[48-50]. It is expected that by analyzing all these parameters during high cell density fed- batch growth of *E. coli* will lead to high volumetric production of the desired protein.

3.2. Important Parameters Affecting Fermentation Process

The operating condition such as pH, temperature and more importantly O_2 supply is very essential for supporting high cell growth. Solubility of oxygen in medium is very low and with increase in cell concentration during fed-batch growth, the solubility is reduced. At very high cell concentration use of air does not suffice the respiratory demand of the rapidly growing *E. coli* cells[51-52]. Increasing aeration rate, feeding O_2- rich air, decreasing temperature, increasing partial pressure of the culture vessel are some of the methods employed to maintain aerobic condition during cell growth[53]. It has been widely documented that oxygen not only influences the cell growth but also has effect on gene expression by influencing the oxidative status of many enzymes[20,54]. Hence it is essential that

along with proper feeding of nutrients, supply of oxygen should be at optimal level to support good growth and provide oxidising environment for quality protein synthesis[19,55-57].

Composition of medium, physical parameters during growth and operating conditions are the most important factors that influence the cell growth. Limitation and or inhibition of substrates, limited capacity of oxygen supply, formation of metabolic byproducts, and instability of plasmid during long hours of cultivation, are the most common problems encountered during high cell density growth of *E. coli*[44,46,58]. These, most of the time depend on host strain, vector, and strength of promoter. Dense culture requires large amounts of O_2 to support good growth and thus necessitates unconventional aeration strategy to maintain dissolved O_2 concentration at a suitable level throughout the growth period. In most of the cases of *E. coli* being used as a host for recombinant protein, the production phase start after induction with suitable inducer[8,40]. Thus in principle, growth phase and production phase can be delineated in the same vessel for the high volumetric yield of the recombinant protein. However many a times, operation of reactor during cell growth influences the specific yield of the recombinant proteins. Thus while developing fed-batch operation to increase unit cell growth in the reactor it is alsoy essential to take care of the factors which affect the specific yield of the recombinant protein.

3.3. Development of Growth Media

The composition of the cell growth medium must be carefully formulated and monitored, because it may have significant metabolic effects on both the cells and protein production. For example, the translation of different mRNAs is differentially affected by temperature as well as changes in the culture medium[59]. Chemically defined media are generally known to produce slower growth and low protein titres than the complex media[33]. Nonetheless, use of chemically defined media in producing recombinant proteins is a common practice[60-63] because these media attain more consistent titres, allow easier process control and monitoring, and simplify downstream recovery of the target protein.

Nutrient composition and fermentation variables such as temperature, p*H*, and other parameters can affect proteolytic activity, secretion, and production levels[47].

Specific manipulations of the culture medium have been shown to enhance protein release into the medium. Thus, supplementation of the growth medium with glycine enhances the release of periplasmic proteins into the medium without causing significant cell lysis[45]. Some nutrients, including carbon and nitrogen sources, can inhibit cell growth when these are present above a certain concentration. This explains why just increasing the concentrations of nutrients in batch culture medium does not yield high cell density. Among the three types of media defined, complex and semi-defined; defined media are generally used to obtain high cell density, as the nutrient concentrations are known and can be controlled during culture. However, semi-defined or complex media are sometimes necessary to boost product formation. To grow cells in a high density, it is necessary to design a balanced nutrient medium that contains all the necessary components in supporting cell growth, avoiding inhibition. It is desirable to make the feed-solution as simple as possible by including sufficient non-carbon and non-nitrogen nutrients in the starting medium. One of the essential requirements during fed-batch operation is to supply nutrients to promote cell growth[18,40]. To limit their toxicity to the growing cells nutrients such as glucose, ammonia, salt are fed approximating their requirement.

Accumulation of nutrients at high concentration inhibits growth and recombinant protein expression[7]. High glucose causes Crabtree effect and leads to accumulation of acetate which is inhibitory to cell growth[40]. In general, most of the media used for high cell growth of *E. coli* have carbon source, mostly glucose; major salts like phosphate, sodium, potassium, magnesium, ammonia and sulphates, iron; minor trace elements and complex nitrogenous compounds. High density growth in general is initiated with low concentration of the most required substrate and the nutrients are added later in the growth period[5,23]. Ideally, components should be added to the fermenter at the same rate at which these are consumed, so that it prevents the nutrient accumulation in toxic levels while promoting good growth. Another factor which needs attention during medium formulation is the solubility of many components, particularly while making concentrated solution for fed-batch addition. High concentration of glycerol, glucose, yeast extract, and trace elements needs careful composition to avoid precipitation.

3.4. Accumulation of Acetate during Fermentation Process

Acetic acid secretion during aerobic growth of *E. coli* is a major constraint of high productivity recombinant fermentation process. *E. coli* excrete 10-30 per cent of the carbon flux from glucose to acetate during its aerobic growth on glucose. Acetic acid decreases both biomass and the expected cell densities. Inhibition of acetate is significant for cells cultured in a defined medium than a complex medium. Inhibitory effect of acetic acid on growth of *E. coli* cells affecting the volumetric productivity of recombinant proteins during high cell density fermentation has been widely documented[1,26-27]. Acetate is produced when *E. coli* is grown under anaerobic or oxygen-limiting conditions; however, *E. coli* cultures growing in the presence of excess glucose can also produce acetate even under aerobic conditions. A high concentration of acetate (> 5 g/l at p*H* 7) reduces growth rate, biomass yield, and maximum attainable cell densities in HCDC. In general, acetate formation is dependent on the medium used and the specific growth rate. The acetate is formed in complex and defined medium when the specific growth rate exceeds 0.2 h^{-1} or 0.35 h^{-1}, respectively. A study with a different *E. coli* strain showed that acetate was produced in a defined medium when the specific growth rate[16,40,46] was higher

than 0.14 h^{-1}. Therefore, the critical specific growth rate that leads to acetate formation varies among strains and depends on the medium used. The production of acetate is greater in fed-batch cultures than in batch cultures owing to the extended culture period. Acetate formation can also be reduced or prevented by altering the formulation of the medium. Acetate is not produced when glycerol is used as a carbon source[16-17,34,64] as well as high cell densities might be achieved relatively easily.

Acetate accumulation affects the substrate utilisation, inhibits cell growth and recombinant protein production, and then it needs special attention during the development of fed-batch fermentation process. A number of strategies have been adopted to reduce the acetate accumulation during high cell density fed-batch cultivation of *E. coil*. Most of the strategies have been based on decreasing anabolic requirements in terms of reducing glucose uptake or increasing the oxidative metabolic capacity[26,65]. Controlling specific growth rate by manipulation of nutrients or nitrogen source have also been tried to reduce the acetate formation[23,40]. Alternatively, the use of dialysis reactor, which facilitates the removal of acetate from the culture broth,[66] can reduce the inhibitory effect of acetate. Genetic engineering techniques, use of fructose as an alternate carbon source, and selection of low-acetate producing strains have been successfully tried to lower the secretion of acetic acid during aerobic growth of *E. coli*. Accumulation of acetate during *E. coli* fermentation is the net result of its formation and uptake by the cells[26]. It's thus essential to control the formation of acetate and enhance its uptake to maintain low level of acetate during fed-batch growth of *E. coli*.

3.5 Effect of Yeast Extract during Fermentation Process

It has been observed that addition of yeast extract not only helps in reducing secretion of acetic acid during growth of *E. coli*, but also helps in utilisation of acetic acid during carbon limitation. Apart from this, organic nitrogen source like yeast extract and soybean hydrolysate have been reported to enhance the specific cellular yield of the expressed protein, particularly during high cell density fermentation where the demand of nitrogenous source becomes very high following induction[30,60]. The use of yeast extract along with glucose feeding helped in maintaining a higher growth rate during fermentation with very little acetic acid secretion. Presence of yeast extract in the medium provides nutrients and precursors for synthesis of building blocks of cells and thus glucose is used mainly as an energy source. Presence of yeast extract in the medium also helps in lowering the inhibitory effect of acetic acid and also works as a better physiological buffer in comparison to the minimal medium[7,34,64]. Therefore, its use in the feeding medium along with glucose helped in avoiding the need of complex genetic manipulation to lower the acetic acid secretion[5]. This indicates that with the use of yeast extract in the feeding medium not only the specific yields of protein can be maintained in high cell density fermentation but also the duration of the process can be reduced, resulting in high volumetric productivity of the expressed protein.

3.6 Feeding Strategy during Fermentation Process

Feeding of nutrient is critical to the success of HCDC, as it not only affects the maximum attainable cell concentration, but also cell productivity[60]. The use of fed-batch cultures has been shown to significantly increase the cell density and specific protein production by overcoming inhibitory substrate concentrations encountered in batch culture[19-20,31,55]. Various strategies exist for controlled feeding of fed-batch cultures. A p*H*-stat feeding system monitors the p*H* of the fermentation for indications of acetic acid production[6,13]. The feed rate is increased until the maximum growth rate is reached as indicated by a metabolic overflow causing production of acid and a consequent decrease in p*H*[25,38]. The DO-stat operation relies on the fact that specific oxygen uptake reaches a maximum at the maximum growth rate[6,13,28]. Changes in oxygen uptake rate following a pulse of feed are used to determine whether the microorganism is at its maximum growth rate[25].

Exponential feeding makes use of an empirical model of growth, to regulate the feed rate[6,12]. One of the essential requirements to obtain good cell growth during cultivation is to supply nutrients in a manner that is desirable. Ideally, by providing proper nutrient and operating conditions, exponential growth of *E. coli* can be maintained so that high cell concentration is achieved in less time. Oxygen supply, saturation of the oxidative capacity of cells at high glucose concentration, build-up of acetate to toxic level, plasmid instability, and low productivity associated with cell growth at high specific growth rate, has led to the development of different feeding strategies to achieve high cell growth in a reasonable time period[19,50,57,65].

Direct feedback control is also possible by measuring online concentration of the growth-limiting substrate in the culture broth and adjusting the concentration to the preset value by automatic feeding. The feeding solution is added either simply in a constant way or by an exponential feeding programme. Two points are important to guarantee a good yield: (a) the flow of the feed solution must be low enough to allow glucose limitation in the fermenter; (b) the flow of the feed solution must be regulated in a way that the specific growth rate does not come below 0.1 h^{-1} until the point of induction[5].

3.7 Plasmid Stability during Fermentation Process

One of the most important factors that affect the expression of recombinant protein from *E. coli* is the maintenance of the plasmid within the host cells[8]. High cell density growth of *E. coli* in fed-batch mode needs longer time and more generation number for cultivation in comparison to normal shaker flask culture. Because of this, plasmid instability problems are serious in large-scale culture[6,8,44]. It is thus essential to have host/vector system as well as cultivation conditions that promote

better plasmid stability during high cell density cultivation. Even though in many cases, the antibiotic pressure is used to overcome plasmid instability, the stability of antibiotic[67] solution during long cultivation period should be taken into consideration. Most of the antibiotics have very short half-life (e.g., Pencillin G or Tetracycline $t_{1/2}$ between 5.6 h and 15.2 h)[67] and it is thus essential to supply them throughout the cultivation period intermittently so the high plasmid stability is maintained during the fed-batch cultivation period[4,58]. Apart from these novel vectors such as run away replication vector (pURA-4) [68], vectors having self-destroying properties of killing plasmidless cells, such as using hok/sok sequences, can be used to overcome the plasmid instability problems[69]. Use of nutrient addition and bioreactor configurations has improved plasmid stability during high cell density fed-batch fermentation[40,44,70].

3.8 Induction Strategy and Effect of Oxygen during Fermentation Process

Cellular responses to induction depend on a number of interacting factors including the host/vector system and properties of the expressed protein. Therefore, the timing of induction of new recombinants needs to be empirically determined for each new clone[61]. Inducer concentration trials are required since IPTG inducer is expensive. The concentration of IPTG required for complete induction is known to vary widely along with various clones expressing different proteins[35]. The induction strategy needs optimisation to maintain specific cellular protein yield during high cell density fed-batch fermentation. Level of gene expression, localisation of the expressed protein, its toxic effect, either due to the gene product or due to high level accumulation of the foreign protein and the product degradation characteristics, decide the induction strategy. For chemical inducer, the concentration of inducer is also important to completely *de-repress* the promoter to achieve maximum protein expression during high cell density fermentation[3,8]. Final synthesis of the protein depends upon the amino acids, thus it is also important to see that the amino acid pools of the host cells meet the demand during high level synthesis of the foreign protein. In fed-batch culture, separation of the two phases can be achieved by delaying induction of the culture until the culture has completed its growth to the required high densities. Considering these aspects, it is essential to decide the induction time so that both cell growth and specific yield are maximised with the result in high volumetric yield of the protein.

In recent times, the secondary role of oxygen on maintenance of cell physiology and quality of the recombinant protein have been a major concern for the high volumetric yield of recombinant protein from *E. coli*[54,71]. Fluctuations in oxygen content during high productive fermentation process can cause oxidative stress within the cells, leading to limitation in amino acid production, plasmid instability, and more importantly, oxidation of proteins. These effects altogether may affect the quality of the final product. Oxygen often becomes limiting in HCDCs owing to its low solubility. The saturated dissolved oxygen (DO) concentration in water at 25°C is ~7mg/l but oxygen supply can be increased by increasing the aeration rate or agitation speed[16]. Oxygen-enriched air or pure oxygen has also been used to prevent oxygen limitation, as for example in high yield production of recombinant malaria antigen and human interferon[18,21]. However, pure oxygen is expensive and increases production costs when used in large quantities. As oxygen consumption increases with growth rate, the oxygen demand of cells can be reduced by lowering the growth rate.

3.9 Metabolic Loads

Some proteins directly influence host cellular metabolism by their enzymatic properties, as the expression of recombinant proteins induces a metabolic burden. The metabolic burden is defined as the amount of resources (raw material and energy) that are required to be withdrawn from the host metabolism for maintenance and expression of the foreign DNA[72]. In general, the specific growth rate of cells expressing a product correlates inversely with the rate of recombinant protein synthesis[73]. The expression of recombinant proteins therefore, usually results in impaired growth rates and lower increase in biomass. This is a direct response to the high energy requirements induced by recombinant protein synthesis, synthesis of stress proteins, and elevated respiration rates[73]. Recombinant expression results in high rates of protein synthesis. However, while the recombinant protein is highly expressed, housekeeping genes, including components of the protein synthesis machinery, are down regulated[72]. Amino acid starvation tends to occur during recombinant expression, if the product deviates considerably from the average *E. coli* protein. If the desired quality and quantity of recombinant protein is impaired, stress situations should clearly be avoided and circumvented.

4. SCALE-UP OF FERMENTATION PROCESS

Recombinant microorganisms are usually cultivated at several cubic meter scale and fed-batch operations have made high cell density fermentations possible, thus increasing the productivity of bioprocesses significantly. A common problem with large-scale protein production is the metabolic overload and stress, since plasmid maintenance and heterologous protein expression are the burden upon the host cell. A robust and efficient aerobic fermentation process requires intense process development and tight control of process parameters such as carbon source concentration, temperature, p*H* and dissolved oxygen[58,74-75].

To increase product yields and to ensure consistent product quality key issues of industrial fermentations process optimisation and scale-up are aimed at maintaining optimum and homogenous reaction conditions, minimising microbial stress exposure, and enhancing metabolic accuracy[44,76]. Cells close to the injection port are exposed to high concentrations of nutrients, whereas at other locations, cells are starved of substrate[4,70]. Thus, it is important to study the mixing patterns in a fermenter, and to find ways

to improve mixing. Therefore, the pressurised culture regime used to increase oxygen transfer may also enhance the detrimental effect of CO_2. Heat generation can also become a problem in HCDC, especially in large-scale fermentations. These problems can be partially solved by growing cells at a reduced specific growth rate. The problem of reduced mixing quality in larger scales is aggravated with increasing vessel sizes: the opposite substrate and oxygen gradients along the vessel height, which are formed as a result of the conventional fermenter design according to which substrate feed usually occurs from the top and aeration from the bottom, are more pronounced in larger reactors due to: (i) longer distances to be covered leading to larger substrate and oxygen depletion zones, (ii) larger volumes of culture broth to be stirred and therewith longer mixing times, and (iii) stronger hydraulic pressure gradients influencing the oxygen-transfer rate[74].

In case of glucose feeding, cells at the fermenter top are exposed to excess glucose concentrations and simultaneously suffer from oxygen imitations, whereas those at the bottom are exposed to glucose starvation. Excess glucose concentrations result in acetate overproduction (overflow metabolism), a simultaneous oxygen limitation further induces the formation of ethanol, hydrogen, formiate, lactate, and succinate (mixed acid fermentation) [20,74]. Amino acid misincorporations are thus a cause for an increase in by-products at the expense of the desired main product yields in larger scales.

Plasmid stability, an essential prerequisite for high product yields particularly in larger scales, in which cultures pass a higher number of generations due to larger culture broth volumes and longer inoculation chains from the cell bank to the production stage, is the stable propagation of plasmids to daughter cells. Plasmid stability is influenced by the plasmid properties[70] as well as by process parameters like temperature, growth rates and substrate concentrations. Rapid glucose oscillations favour plasmid stability and recombinant protein production rate, whereas high glucose concentrations diminish plasmid stability[65]. Plasmid stability and plasmid numbers are thus negatively influenced, more difficult to control, and less easy to be maintained in larger scales. A prioritised goal of process optimisation and scale-up thus consists of an appropriate process design which improves the physiological conditions and the metablic accuracy by minimising microbial stress exposure.

5. CONCLUSIONS AND FUTURE CHALLENGES

Although many alternative organisms and expression systems are now being used for recombinant protein production, exciting progress continues to be made with *E. coli*. Recombinant proteins in *E. coli* can be produced to high yield by fed-batch culture. It is now relatively easy to obtain high protein yield by controlling the acetate concentration below the inhibitory level which allows the control of the specific growth rate. While glucose or glycerol serves as carbon sources, yeast extract, plant-derived hydrolysates or peptones are usually employed as complex nitrogen sources. Complex media components may be critical since these provide a source for process variability. An increasing number of recombinant protein diagnostics as well as therapeutics will have to be produced in the years to come.

Microbial expression systems, e.g. *E. coli* will aid in efficient production of bio-pharmaceuticals. Major constraints of high productive recombinant protein expression during aerobic growth of *E. coli* are the secretion of acetic acid, effect of medium, dissolved oxygen, lowering of the specific cellular protein yield, etc. To avoid secretion of acetic acid, cells are generally grown at lower specific growth rates to achieve high cell concentration. Operation of fed-batch fermentation at lower specific growth rate of *E. coli* extends the duration of the batch time, and hence, affects volumetric productivity of recombinant protein. Future challenges in the use of *E. coli* for high level expression will involve the following factors.

- The first one is the achievement of enhanced yields of desired proteins by maintaining desired parameters, viz., dissolved oxygen, p*H*, temperature, agitation, feeding rate of nutrients.
- The second one is the utilisation of carbon and nitrogen sources at controlled rates.
- It would be interesting to investigate, in the future, if one has also to look for new alternative methods to improve the overall yields and quality of desired product economically.
- Another challenge is to overcome the drawbacks during fermentation process.

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REFERENCES

1. Yee, L. & Blanch, H.W. Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Bio/Technology*, 1992, **10**,1150-56.
2. Akesson, M.; Hagander, P. & Axelsson, J.P. Probing control of fed-batch cultivations: analysis and tuning. *Control Eng. Practice*, 2001, **9**, 709-23.
3. Baneyx, F. Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.*, 1999, **10**, 411-21.
4. Jana, S. & Deb, J.K. Strategies for efficient production of heterologous proteins. *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, 2005, **67**, 289-98.
5. Fahnert, B.; Lilie, H. & Neubauer, P. Inclusion bodies: formation and utilisation. *Adv. Biochem. Eng. Biotechnol.*, 2004, **89**, 93-142.
6. Yee, L. & Blanch, H.W. Recombinant trypsin production in high cell density fed-batch cultures of *Escherichia coli*. *Biotechnology Bioengineering*, 1993, **41**,781-90.

7. Shiloach, J.& Fass, R. Growing *E. coli* to high cell density–A historical perspective on method development. *Biotechnology Advances*, 2005, **23**, 345-57.
8. Akesson, M.; Hagander, P. & Axelsson, J.P. Probing control of fed-batch cultivations: analysis and tuning. *Control Eng. Practice*, 2001, **9**, 709-23.
9. Das, A. Overproduction of proteins in *Escherichia coli*: vectors, hosts, and strategies. *Methods Enzymol.*,1990, **182**, 93-112.
10. Hewitt, L. & McDonnell, J.M. Screening and optimizing protein production in *E. coli*. *Meth. Mol. Biol.*, 2004, **278**, 1–16.
11. Korz, D.J.; Rinas, U.; Hellmuth, K.; Sanders, E.A. & Deckwer, W.D. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *Journal of Biotechnology*, 1995, **39**, 59-65.
12. Lee, J.; Lee, S.Y. & Park, S. Fed-batch culture of *Escherichia coli* by exponential feeding of sucrose as a carbon source. *Biotechnol. Techniq.*, 1997, **11**, 59-62.
13. Jeong, K.J. & Lee, S.Y. High-level production of human leptin by fed-batch cultivation of recombinant *Escherichia coli* and its purification. *Appl. Environ. Microbiol.*, 1999, **65,** 3027-032.
14. Jeong, K.J.; Choi, J.H.; Yoo, W.M.; Keum, K.C.; Yoo, N.C.; Lee, S.Y. & Sung, M.H. Constitutive production of human leptin by fed-batch culture of recombinant *rpoS-Escherichia coli*. *Protein Exp. Purific.* 2004, **36**, 150-56.
15. Wu, P.H.; Nair, G.R.; Chu, I.M. & Wu, W.T. High cell density cultivation of *Escherichia coli* with surface anchored transglucosidase for use as whole-cell biocatalyst for alpha-arbutin synthesis. *J. Ind. Microbiol. Biotechnol.*, 2008, **35**, 95-101.
16. Lee, S.Y. High cell-density culture of *Escherichia coli*. *Trends Biotechnol.* 1996, **14**, 98-105.
17. Riesenberg, D. & Guthke, R. High-cell-density cultivation of microorganisms. *Appl. Microbiol. Biotechnol.*, 1999, **51**, 422-30.
18. Khalilzadeh, R.; Shojaosadati, S.A.; Maghsoudi, N.; Mohammadian-Mosaabadi, J.; Mohammadi, M. R.; Bahrami, A.; Maleksabet, N.; Nassiri-Khalilli, M.A.; Ebrahimi, M. & Naderimanesh, E.H. Process development for production of recombinant human interferon-*c* expressed in *E. coli*. *J. Ind. Microbiol. Biotechnol.*, 2004, **31**, 63-69.
19. Chen, Q.; Bentley, W.E & Weigand,W.A. Optimisation for a recombinant *E. coli* fed-batch fermentation. *Appl. Biochem.Biotechnol.* 1995, **51**,449-61.
20. Castan, A. & Enfors, S.O. Characteristics of a DO-controlled fed-batch culture of *Escherichia coli*. *Bioproc. Eng.* 2000, **22,** 509-15.
21. Yazdani, S.S.; Shakri, A.R. & Chitnis, C.E. A high cell density cultivation strategy to produce recombinant malarial antigen in *Escherichia coli*. *Biotechnol. Lett.* 2004, **26**, 1891-895.
22. Clementschitsch, F.; Kern, J.; Pötschacher, F& Bayer, K. Sensor combination and chemometric modeling for improved process monitoring in recombinant *E. coli* fed-batch of an antibody Fab fragment cultivations. *J. Biotechnol.* 2005, **120**, 183-96.
23. Babaeipour, V.; Shojaosadati, S.A.; Khalilzadeh, R.; Maghsoudi, N. & Tabandeh, F. A. proposed feeding strategy for the overproduction of recombinant proteins in *Escherichia coli*. *Biotechnol. Appl. Biochem.*, 2008, **49**, 141-47.
24. Turner, C.; Gregory, M.E. &Turner, M.K. A study of the effect of specific growth-rate and acetate on recombinant proteinproduction of *Escherichia coli* JM107. *Biotechnology Letters*. 1994, **16**, 891-96.
25. Akesson, M.; Hagander, P. & Axelsson, J. Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. *Biotechnology Bioengineering*. 2001, **73,** 223-30.
26. Eiteman, M.A. & Altman, E.Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends in Biotechnology*. 2006, **24**, 530-6.
27. De Mey, M.; De Maeseneire, S.; Soetaert, W. & Vandamme, E. Minimizing acetate formation in *E. coli* fermentations. *J. Ind. Microbiol. Biotechnol.* 2007, **34**, 689-700.
28. Akesson, M.; Karlsson, E.N.; Hagander, P.; Axelsson, J. & Tocaj, A. On-line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. *Biotechnology Bioengineering*. 1999, **64,** 590-98.
29. Li, Y.; Chen, J.; Mao, Y.; Lun, S. & Koo, Y. Effect of additives and fed-batch culture strategies on the production of glutathione by recombinant *Escherichia coli*. *Process Biochem.* 1998, **33**, 709-14.
30. Manderson, D.; Dempster, R. & Chisti, Y. Recombinant vaccine against hydatidosis: production of the antigen in *Escherichia coli*. *J. Ind. Microb. Biotechnol.* 2006, **33**, 173-182.
31. Tripathi, N.K.; Srivastva, A. & Rao, P.V.L. Process development for production of recombinant protective antigen from *Bacillus anthracis*. *Ind. Chemical Engr.* 2006, **48**,178-84.
32. Lim, H.K.; Jung, K.H.; Park, D.H. & Chung, S.I. Production characteristics of interferon-a using an l-arabinose promoter system in a high-cell-density culture. *Appl. Microbiol. Biotechnol.*, 2000, **53,** 201-08.
33. Zanette, D.; Dundon, W.; Soffientini, A.; Sottani, C.; Marinelli, F.; Akeson, A. & Sarubbi, E. Human IL-1 receptor antagonist from *Escherichia coli*: large-scale microbial growth and protein purification. *Journal of Biotechnology*. 1998, **64**,187-96.
34. Tripathi, N.K.; Babu, JP.; Shrivastva, A.; Parida, M.; Jana, A.M. & Rao, P.V.L. Production, and characterization of recombinant dengue virus type 4 envelope domain III protein. *Journal of Biotechnology* 2008, **134**, 278-86.
35. Madurawe, R.D.; Chase, T.E.; Tsao, E.I. & Bentley, W.E. A recombinant lipoprotein antigen against Lyme disease expressed in *E. coli*: fermentor operating strategies for improved yield. *Biotechnology Progress*. 2000,

16, 571–76.

36. Tripathi, N.K.; Shrivastva, A.; Pattnaik, P.; Parida, M.M.; Dash, P.K.; Gupta, N.; Jana, A.M. and Rao, P.V.L. Production of IgM specific recombinant dengue multiepitope protein for early diagnosis of dengue infection. *Biotechnology Progress,* 2007, **23**, 488-93.
37. Tripathi, N.K.; Saxena, P.; Sathyaseelan, K. and Rao, P.V.L. Production, of recombinant dengue virus type 2 envelope domain III protein in *Escherichia coli*. *Ind. Chem. Engr*., 2008, (In press).
38. Studier, F.W.; Rosenberg, A.H.; Dunn, J.J. & Dubendorff, J.W. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.,* 1990, **185**, 60-89.
39. Ray, M.V.; Meenan, C.P.; Consalvo, A.P.; Smith, C.A.; Parton, D.P.; Sturmer, A.M.; Shields, P.P. & Mehta, N.M. production of salmon calcitonin by direct expression of a glycine-extended precursor in *Escherichia coli*. *Protein Expr. Purif.,* 2002, **26**, 249-59.
40. Panda, A.K. Bioprocessing of therapeutic proteins from the inclusion bodies of *Escherichia coli*. Adv. Biochem. Eng. Biotechnol., 2003, **85**, 43-93.
41. Georgiou, G. & Segatori, L. Preparative expression of secreted proteins in bacteria: Status report and future prospects. *Curr. Opin. Biotechnol.*, 2005, **16**, 538-45.
42. Stanbury, P.F.; Whitaker, A. & Hall, S.J. Principles of Fermentation Technology, Ed. 2. UK, Elsevier Science Ltd, 2005.
43. Gupta. P.; Sahai, V. & Bhatnagar, R. Enhanced expression of the recombinant lethal factor of *Bacillus anthracis* by fedbatch culture. *Biochem. Biophys. Res. Com*. 2001, **285**,1025-033.
44. Schmidt F.R. Optimisation and scale-up of industrial fermentation processes. *Appl. Microbiol. Biotechnol.* 2005, **68**, 425-35.
45. Aristidou, A.A.; San, K.Y. & Bennett, G.N. Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. *Biotechnology Progress.*, 1995, **11,** 475-78.
46. Luli, G.W. & Strohl, W.R. Comparison of growth, acetate production and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.*, 1990, **56**, 1004-011.
47. Bird, P. I.; Pak, S. C.; Worrall, D. M.; Stephen, P. & Bottomley, S. P. Production of recombinant serpins in *Escherichia coli*. *Methods,* 2004, **32**,169-76.
48. Zhang, X.W. ; Sun, T. ; Liu, X. ; Gu, D.X. & Huang, X.N. Human growth hormone production by high cell density fermentation of recombinant *Escherichia coli*. *Process Biochem.* 1998, **33**, 683-86.
49. Schmidt, M.; Babu, K.R.; Khanna, N.; Marten, S. & Rinas, U. Temperature-induced production of recombinant human insulin in high-cell density cultures of recombinant *E. coli. Journal of Biotechnology.* 1999, **68**, 71-83.
50. Babu, K. R.; Swaminathan, S.; Marten, S.; Khanna, N. & Rinas, U. Production of interferon-a in high cell density cultures of recombinant *Escherichia coli* and its single-step purification from re-folded inclusion body proteins. *Appl. Microbiol. Biotechnol*., 2000, **53**, 665-60.
51. Bhattacharya, S. K.&. Dubey, A. K. Effect of dissolved oxygen and oxygen mass transfer on overexpression of target gene in recombinant *E. coli*. *Enzyme Microb. Technol.*, 1997, **20,** 355-60.
52. De Leon, Hernandez, V.; Galindo, E. & Ramirez, O.T. Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli*. *Enzyme Microb. Tech.,* 2003, **33**, 689-97.
53. Franz, C.; Jurgen, K.; Florentina, P. & Karl, B. Sensor combination and chemometric modelling for improved process monitoring in recombinant *E. coli* fed-batch cultivations. *Journal of Biotechnology.* 2005, **120**, 183-96.
54. De Mare, L.; Velut, S.; Ledung, E.; Cimander, C; Norrman, B.; Nordberg Karlsson, E.; Holst, O. & Hagander, P. A cultivation technique for *E. coli* fedbatch cultivations operating close to the maximum oxygen transfer capacity of the reactor. *Biotechnology Letters*. 2005, **27**, 983-90.
55. Chen, W.; C. Graham, C & Ciccarelli, R.B. Automated fed-batch fermentation with feed-back controls based on dissolved oxygen (DO) and p*H* for production of DNA vaccines. *J. Ind. Microbiol. Biotech.*,1997, **18,** 43-48.
56. Whifin, V.S.; Cooney, M.J. & Cord-Ruwisch, R. Online detection of feed demand in high cell density cultures of *Escherichia coli* by measurement of changes in dissolved oxygen transients in complex media. *Biotechnology Bioengineering*. 2004, **85**, 422-33.
57. Phue, J.N. & Shiloach, J. Impact of dissolved oxygen concentration on acetate accumulation and physiology of *E. coli* BL21, evaluating transcription levels of key genes at different dissolved oxygen conditions. *Metab. Eng.*, 2005, **7**, 353-63.
58. Johnston, W.R.; Cord-Ruwisch & Cooney, M.J. Industrial control of recombinant *E. coli* fed-batch culture: new perspectives on traditional controlled variables. *Bioproc. Biosyst. Eng.*, 2002, **25**, 111-20.
59. Corisdeo, S & Baiyang, Wang, B. Functional expression and display in *Escherichia coli*: study of vector designs and culture conditions. *Prot. Exp. Purif.*, 2004, **34**, 70-279.
60. Lim, H.K. & Jung, K.H. Improvement of heterologous protein productivity by controlling post induction specific growth rate in recombinant *Escherichia coli* under control of the PL promoter. *Biotechnology Progress*, 1998, **14**, 548-53.
61. Cserjan-Puschmann, M.; Kramer, W.; Duerrschmid, E.; Striedner, G. & Bayer, K. Metabolic approaches for the optimisation of recombinant fermentation processes. *Appl. Microbiol. Biotechnol*., 1999, **53,** 43-50.
62. Zhang, J. & Greasham, R. Chemically-defined media for commercial fermentations. *Appl. Microbiol. Biotechnol.*, 1999, **51**, 407- 21.
63. Kweon, D.H.; Han, N.S.; Park, K.M. & Seo, J.H.

Overproduction of *Phytolacca insularis* protein in batch and fed-batch culture of recombinant *Escherichia coli*. *Processes in Biochemistry.,* 2001, **36**, 537-42.

64. Tripathi, N.K.; Shrivastva, A.; Pattnaik, P.; Parida, M.; Dash, P.K.; Jana, A.M. & Rao, P.V.L. Production, purification and characterization of recombinant dengue multiepitope protein. *Biotechnol. Appl. Biochem.*, 2007, **46**, 105-13.
65. Lin, H.Y. & Neubauer, P. Influence of controlled glucose oscillations on a fed-batch process of recombinant *Escherichia coli*. *Journal of Biotechnology,* 2000, **79**, 27-37.
66. Landwall, P. & Holme, T. Influence of glucose and dissolved oxygen concentrations on yields of *Escherichia coli* in dialysis culture. *J. Gen. Microbiol.*, 1997, **103**, 353-58.
67. Peteranderl, R.; Shotts, E.B. Jr. & Wiegel, J. Stability of antibiotics under growth conditions for thermophilic anaerobes. *Appl. Environ. Microbiol.*, 1990, **56**, 1981-983.
68. Trepod, C. & Mott, J. A spontaneous runaway vector for production scale expression of bovine somatotropin from *Escherichia coli. Appl. Microb. Biotechnol.*, 2002, **58**, 84-88.
69. Friehs, K. & Reardon, K.F. Parameters influencing the productivity of recombinant *E. coli* cultivations. *Adv. Biochem. Eng. Biotechnol.*, 1993, **48**, 53-77.
70. Thiry, M. & Cingolani, D. Optimzing scale-up fermentation processes. *Trends in Biotechnology.* 2002, **20**, 103-05.
71. Konz, J.O.; King, J. & Conney, C.L. Effect of oxygen on recombinant protein expression. *Biotechnology Progress,* 1998, **14**,393-409.
72. Sorensen, H.P. & Mortensen, K.K. Advanced genetic strategies for recombinant protein expression in *Eschericia coli*. *Journal of Biotechnology,* 2005, **115**, 113-28.
73. Hoffmann, F. & Rinas, U. Stress induced by recombinant protein production in *Eschericia coli*. *Adv. Biochem. Eng. Biotechnol.*, 2004, **89**, 73-92.
74. Bylund, F.; Castan A.; Mikkola, R.; Veide, A. & Larsson, G. Influence of scale-up on the quality of recombinant human growth hormone. *Biotech. Bioeng.* 2000, **69**, 119-28.
75. Kavanagh, J.M. & Barton, G.W. Productivity improvement of recombinant *Escherichia coli* fermentation via robust optimisation. *Bioprocess Biosyst. Eng.,* 2008, **31**,137-43.
76. O'Connor, G.M.; Sanchez-Riera, F. & Cooney, C.L. Design and evaluation of control strategies for high cell density fermentations. *Biotechnol. Bioeng.* 1992, **39**, 293-04.