Review article

Recent advances in high cell density cultivation for production of recombinant protein

Seyed Abbas Shojaosadati[*1], Seyedeh Marjan Varedi Kolaei[1,2], Valiollah Babaeipour[1], Amir Mohammad Farnoud[1]

[1]Biotechnology Group, Department of Chemical Engineering, Faculty of Engineering, Tarbiat Modares University, P.O. Box 14115-143, Tehran, I.R. Iran [2]Department of Biotechnology, University College of Science, University of Tehran, P.O. Box 14155-6455, Tehran, I.R. Iran

Abstract
This paper reviews recent strategies used for increasing specific yield and productivity in high cell density cultures. High cell density cultures offer an efficient means for the economical production of recombinant proteins. However, there are still some challenges associated with high cell density cultivation (HCDC) techniques. A variety of strategies in several aspects including host design consideration, tuning recombinant protein expression, medium composition, growth methodologies, and even control and analysis of the process have been successfully employed by biotechnologists to increase yield in high cell density cultures. Although most researches have focused on *Escherichia coli*, other microorganisms have the potential to be grown at high density and need further investigation. In recent years, information on physiological changes of hosts during different phases of cultivation derived from functional genomics, transcriptomics and proteomics is being used to overcome the obstacles encountered in high cell density cultivation and hence increase productivity.
Keywords: High cell density culture; Recombinant protein; Expression system.

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1. INTRODUCTION

High cell density cultivation (HCDC) is a powerful technique for production of recombinant proteins, the annual market growth of which is expected to increase at a rate of 10-15% per annum (Werner, 2004). The combination of large scale culture processes with recombinant DNA technology has enabled proteins such as interferons, interleukins, colony-stimulating factors and growth hormones to be produced in quantities that might otherwise have been difficult, if not impossible, to obtain from natural sources. Productivity is a function of cell density and specific productivity (i.e. the amount of product formed per unit cell mass per unit time); so increasing the cell density as well as specific productivity increases productivity. Increasing productivity is the major objective of fermentation in research and industry and as metioned by Lee (1996) and Riesenberg and Guthke (1999),

[*]*Correspondence to:* **Seyed Abbas Shojaosadati,** Ph.D.
Tel: +98 21 82883341; Fax: +98 21 82883381
E-mail : shoja_sa@modares.ac.ir

Table 1. Advantages and disadvantages of HCDC (Choi et al., 2006; Kleman and Strohl, 1994; Lee, 1996; Riesenberg and Guthke, 1999).

HCDC	
Advantages	Disadvantages
Increased cost effectiveness	Substrate inhibition or limitation
Reduced culture volume	Limited transfer and high demand of oxygen
Easier downstream processing	Cell lysis and proteolysis
Reduced investment in equipments	Limited heat transfer
Reduced waste water	Formation of growth inhibitory byproducts
-	Plasmid instability
-	High production rates of CO_2 and heat

HCDCs are a prerequisite to maximize the amount of product in a given volume within a certain time. HCDC enables the researchers to reach a higher dry cell weight and as a result a higher product concentration which is not possible in conventional batch and continuous processes. So far, an exact dry cell weight per liter has not been considered as a representative of high cell density and different studies have considered different values of dry cell weight like 50 g/l (Shokri and Larsson, 2004; Rozkov, 2001) and even values in the range of 20 g/l for a culture to be named HCDC.

The first step for producing protein in HCDC systems is choosing a suitable expression system, well adapted to HCDC. Once the expression system is developed, fermentation is carried out to increase the protein product titer. Nutrient composition, feeding strategy and growth conditions should be optimized in order to reach HCDC. The advantages and disadvantages of HCDC are mentioned in Table 1. It should be mentioned that despite such disadvantages, the economical advantages of HCDC over conventional methods of fermentation are often so great that it is usually just a matter of how to overcome these disadvantages and set up a HCDC. However, for large-scale processes concerns like using pure oxygen, pressurized bioreactor, high mechanical load on the agitation system and sensing and probing limitations should also be considered (Shiloach and Fass, 2005).

This review focuses on various approaches and recent advances in solving the problems associated with HCDC and increasing productivity via increasing cell density and/or specific productivity.

2. Expression system improvement: Although, most HCDCS are associated with *Escherichia coli* as listed by Choi *et al*. (2006), other microorganisms have the ability to be grown to high cell densities (Table 2). For example, bacteria such as *Bacillus subtilis* (Vuolanto *et al*., 2001), *Lactobacillus plantarum* (Barreto *et al*., 1991), *Pseudomonas putida* (Lee *et al*., 2000), *Methylobacterium extorquens* (Belanger *et al*., 2004), *Ralstonia eutropha* (Srinivasan *et al*., 2003), yeasts such as *Saccharomyces cerevisiae* (Shang *et al*., 2006), *Kluyveromyces marxianus* (Hensing *et al*., 1994), *Pichia pastoris* (Daly and Hearn, 2005), *Hansenula polymorpha* (Moon *et al*., 2004), *Trigonopsis variabilis* (Kim *et al*.,1997), insect cells like *Spodoptera frugiperda* (Elias *et al*., 2000), animal cells like Chinese hamster ovary cells (Lim *et al*., 2006), diatom *Nitzschia laevis* (Wen *et al*., 2002), Protozoon *Colpidium campylum* (Scheidgen-Kleyboldt *et al*., 2003), *Tetrahymena thermophila* (Kiy and Tiedtke, 1992) and even herbs such as *Panax notoginseng* (Zhong *et al*., 1999) and *Galdieria sulphuraria* (Schmidt *et al*., 2005) and other eukaryotic cells have been reported which can grow to a high cell density.

Microorganisms frequently experience different kinds of limiting conditions during HCDC. Cells in high density cultures are exposed to adverse conditions such as lack of nutrients, elevated osmotic pressure and other problems which have been mentioned previously, so selecting and designing a suitable host with a higher specific growth rate, increased biomass yield, reduced secretion of overflow metabolites and increased resistance to osmotic stress and nutrient deprivation is the primary step in designing a HCDC for producing recombinant proteins.

The traditional approach for obtaining a suitable host is isolation and selection of mutants. Weikert *et al*. (1998) reported a three fold increase in expressing *Bacillus stearothermophilus* amylase using the *E. coli* mutant CWML2:pCSS4-p which had been isolated

Table 2. Different microorganisms used for HCDC and production of recombinant proteins, their products and methodologies.

Host	Cell Density	Product	Productivity and characteristics	Growth & Feeding Strategy	References
Bacillus subtilis					
Bacillus subtilis	56 g/l	Phytase	47.7 U/ml	Fed batch (Controlled glucose concentration)	Vuolanto *et al.* (2001)
Bacillus subtilis	35.6 g/l	Phytase	28.7 U/ml	Fed batch (Controlled glucose concentration)	Kerovuo *et al.* (2000)
Bacillus subtilis	OD 60.1	Penicillin G acylase	1960 U/l	Fed batch (pH-stat)	Zhang *et al.* (2006)
Bacillus subtilis	OD 65	Subtilisin	6.19 U/l	Fed batch (exponential feeding)	Oh *et al.* (2002)
Bacillus subtilis	184 g/l	β-galactosidase	157 g/l.day	Fed bach (pH-stat)	Park *et al.* (1992)
Saccharomyces cerevisiae					
Saccharomyces cerevisiae	~ 45 g/l	Cutinase	1.6 g/l	Fed batch (pH-stat)	Ferreira *et al.* (2004a)
Saccharomyces cerevisiae	~ 45 g/l	Human granulocyte-colony stimulating factor	1.3 g/l	Fed batch (pH-stat)	Lee *et al.* (1999)
Saccharomyces cerevisiae	120 g/l	Ergosterol	1500 mg/l	Fed batch (Controlled glucose concentration)	Shang *et al.* (2006)
Saccharomyces cerevisiae	53.6 g/l	Human granulocyte-colony stimulating factor	~150 mg/l	Fed batch (pH-stat)	Bae *et al.* (1999)
Saccharomyces cerevisiae	50 g/l	β-galactosidase	6.28*10^5 U/lh	Continuous	Dominguez *et al.* (2005)
Pichia pastoris					
Pichia pastoris	125 g/l	Human cystatin_C	0.72 g/l	Fed batch (mixed feeding)	Files *et al.* (2001)
Pichia pastoris	325 g/l (WCW)	Human regenerating gene IV	25 mg/l	Fed batch (DO-stat)	Li *et al.* (2003)
Pichia pastoris	110 g/l	α-amylase	340 mg/l	Fed batch (DO-stat)	Lee *et al.* (2003)
Pichia pastoris	OD 321	Phytase	4946 U/ml	Fed batch (DO-stat)	Chen *et al.* (2004)
Pichia pastoris	~ 45 g/l	Bovine entrokinase light chain	9000 U/ml	Fed batch (pH-stat)	Peng *et al.* (2004)
Ralstonia eutropha					
Ralstonia eutropha	113 g/l	Organophosphohy drolase	1353 U/mg	Fed batch (changing the feed solution)	Barnard *et al.* (2004)
Ralstonia eutropha	182 g/l	Organophosphohy drolase	1.2 g/l	Fed batch (changing the feed solution)	Srinivasan *et al.* (2003)
Ralstonia eutropha	281 g/l	Poly(3-hydroxybutyrate)	232 g/l	Fed batch (DO-stat)	Ryu *et al.* (1997)
Ralstonia eutropha	75 g/l	Poly(3-hydroxybutyrate)	54.8 g/l	Fed batch (pH-stat)	Tsuge *et al.* (2001)
Ralstonia eutropha	300 g/l	Poly(3-hydroxybutyrate)	97% recovery	Fed batch (DO-stat)	Kim *et al.* (2003)
Hansenula polymorpha					
Hansenula polymorpha	~120 g/l	Levansucrase	12200 U/l	Fed batch (DO-stat)	Park *et al.* (2004)
Hansenula polymorpha	~35 g/l	Hirudin	144 mg/l	Fed batch (DO-stat)	Kim *et al.* (1998)
Hansenula polymorpha	83 g/l	Human serum albumin	550 mg/l	Fed batch (intermittent feeding)	Heo *et al.* (2003)
Hansenula polymorpha	350 g/l (WCW)	Human α1 (I) procollagen	0.6 g/l	Fed batch (changing the feed solution)	de Bruin *et al.* (2000)
Hansenula polymorpha	OD 70	Interferon α-2a	350 mg/l	Fed batch (pH-stat, DO-stat)	Muller *et al.* (2002)

Table 2. Continued.

Kluyveromyces marxianus					
Kluyveromyces marxianus	105 g/l	Biomass	2.9 g/l.h	Fed bacth (exponential feeding)	Bojorge *et al*. (1999)
Kluyveromyces marxianus	28.13 g/l	Oligonucleoides	2.42 g/l.h	Fed bacth (controlled lactose concentration)	Ferreira *et al*. (2004b)
Kluyveromyces marxianus	~60 g/l	Lactase	N/A	Fed batch (pH-stat)	Belem & Lee (1999)
Kluyveromyces marxianus	~20 g/l	β-galactosidase	~3000 U/g	Fed batch (stepwise nutrient feeding)	Lukondeh *et al*. (2005)
Kluyveromyces marxianus	12.2 g/l	β-galactosidase	2800 U/g	Batch	Cortes *et al*. (2005)
Panax notoginseng					
Panax notoginseng	27.3 g/l	Ginseng saponin	290 mg/l	Fed batch (combined feeding)	Wang *et al*. (2005)
Panax notoginseng	28.9 g/l	Ginseng saponin	0.92 g/l	Batch	Zhong *et al*. (1999)
Panax notoginseng	24 g/l	Ginseng saponin	1.75 g/l	Batch	Han & Zhong (2003)
Panax notoginseng	24 g/l		1.7 g/l	Batch	
	30 g/l	Ginseng saponin	2.3 g/l	Fed batch (SOUR based feeding)	Han & Zhong (2002)
Panax notoginseng	24.1 g/l		1.4 g/l	Batch	
	29.7 g/l	Ginseng saponin	2.1 g/l	Fed batch (SOUR based feeding)	Hu *et al*. (2001)
Taxus chinensis cells					
Taxus chinensis cells	22 g/l	Paclitaxel	67 mg/l	Fed batch (intermittent feeding)	Choi *et al*. (2000)
Taxus chinensis cells	22.7 g/l	Taxane	278 mg/l	Fed batch (combined feeding)	Dong & Zhong (2002)
Taxus chinensis cells	16.58 g/l	Taxane	5.4 mg/DCW	Batch	Pan *et al*. (2000)
Taxus chinensis cells	15.9 g/l	Taxoid	~70 mg/l	Batch	Zhong *et al*. (2002)
Taxus chinensis cells	~17 g/l	Taxol	67.1 mg/l	Batch	Wang *et al*. (2001)
Spodoptera frugiperda					
Spodoptera frugiperda	5.2*10^7 cells/ml	β-galactosidase	500 U/ml	Fed batch (pulse & semi continuous feedings)	Elias *et al*. (2000)
Spodoptera frugiperda	7*10^6 cells/ml	L1	200 mg/l	Batch	Gouveia *et al*. (2007)
Spodoptera frugiperda	7-9*10^6 cells/ml	Bovine rhodopsin	4 mg/l	Batch	Klaassen *et al*. (1999)
Spodoptera frugiperda	3*10^7 cells/ml	Human chitinase	N/A	Perfusion culture	Zhang *et al*. (1998)
Spodoptera frugiperda	8*10^ cells/ml	β-galactosidase	5-9.8*10^5 U/ml	Batch	Radford *et al*. (1997)
Tetrahymena thermophila					
Tetrahymena thermophila	54 g/L	N/A	N/A	Continuous	Kiy and Tiedtke (1992)
Tetrahymena thermophila	2.2*10^7 cells/ml (48 g/l)	Some lysosomal enzymes	Different	Continuous with cell recycling	Kiy *et al*. (1996)
Tetrahymena thermophila	48 g/L	Human DNase I	100 µg/L	Batch	Weide *et al*. (2006)
Tetrahymena thermophila	1.9*10^6 cells/ml	Protease	~900 mU/ml	Continuous with cell recycling	de Coninck *et al*. (2000)
Tetrahymena thermophila	3*10^6 cells/ml	Protease	~400 nkat/l	Batch	Hellenbroich *et al*. (1999)

Table 2. Continued.

Chinese hamster ovary cells					
Chinese hamster ovary cells	6.2*10^6 cells/ml	Interferon α	~20 mg/l	Fed batch (exponential feeding)	Lim *et al*. (2006)
Chinese hamster ovary cells	5*10^6 cells/ml	Human Antithrombin	1g/l	Fed batch (controlled glucose concentration)	Kuwae *et al*. (2005)
Chinese hamster ovary cells	0.1*10^6 cells/ml	N/A	N/A	Perfusion	Meuwly *et al*. (2006)
Chinese hamster ovary cells	3.71×10^6 cells/ml	Erythropoietin	121.2 μg/mL	Biphasic culture	Yoon *et al*. (2006)
Chinese hamster ovary cells	2*10^9 cells/ml	Angiostatin-human IgG	130 mg/l	Semi-batch	Wang *et al*. (2005)
Escherichia coli					
Escherichia coli	115 g/l	Human interferon γ	42.5 g/l	Fed batch (modified variable specific growth rate)	Babaeipour *et al*. (2007)
Escherichia coli	101 g/l	Poly(3-hydroxybutyrate)	4.4 g/l	Fed batch (exponential feeding and pH-stat)	Kim *et al*. (2004)
Escherichia coli	~90 g/l	Human interferon α	4 g/l	Fed batch (exponential feeding)	Babu *et al*. (2000)
Escherichia coli	162 (WCW)	Penicillin G Acylase	37 IU/WCW	Fed batch (controlled sorbitol concentration)	Liu *et al*. (2000)
Escherichia coli	80 g/l	Gluthathione	880 mg/l	Fed batch (exponential feeding)	Li *et al*. (1998)
Other microorganisms					
Methylobacterium extorquens	56 g/l	Green fluorescent protein	4 g/L	Fed batch (OTR based feeding)	Belanger *et al*. (2004)
Methylobacterium extorquens	100-115 g/l	Poly(3-hydroxybutyrate)	0.2 g/DCW	Fed batch (OTR based feeding)	Bourque *et al*. (1995)
Methylobacterium extorquens	233 g/l	Poly-β-hydroxy butyric acid	32.9 g/l.day	Fed batch (DO-stat)	Suzuki *et al*. (1986)
Nitzschia laevis	~25 g/l	Eicosapentaenoic acid	321 mg/l.day	Perfusion-cell bleeding culture	Wen and Chen (2001)
Nitzschia laevis	22.1 g/l	Eicosapentaenoic acid	695 mg/l	Fed batch (glucose controlled)	Wen *et al*. (2002)
Nitzschia laevis	40 g/l	Eicosapentaenoic acid	1112mg/l	Perfusion culture	Wen and Chen (2002)
Bacillus clausii	~60 g/l	Savinase	15 mg /DCW	Fed-batch (linear and exponential feeding)	Christiansen *et al*. (2003)
Trigonopsis variabilis	34 g/l	Erythritol	46 g/l	Two stage fermentation	Kim *et al*. (1997)
Crypthecodinium cohnii	109 g/L	Docosahexaenoic acid	19 g/L	Fed batch (DO-stat)	De Swaaf *et al*. (2003)
Galdieria sulphuraria	80-120 g/L	Phycocyanin	250-400 mg/l	Fed batch (DO-stat)	Schmidt *et al*. (2005)
Pseudomonas putida	34 g/l	Di-heme protein cytochrome c(4)	29 mg/l	Fed batch (exponential feeding)	Thuesen *et al*. (2003)

WCW: Wet Cell Weight
DCW: Dry Cell Weight
N/A: Not Applicable
DO: Dissolved Oxygen
OTR: Oxygen Uptake Rate
SOUR: Specific Oxygen Uptake Rate

from a mixed culture.

Powerful tools of genetics and cellular engineering have enabled researchers to design a better host for HCDC by rational instead of trial-and-error methods. Jena and Deb (2005) and Sorensen *et al*. (2005) listed genetic parameters to be considered for designing a better expression system. Moreover, redirecting the metabolic pathways has become more common recently. Especially that proteome and transcriptome profiling of microorganisms make it possible to generate invaluable information that can be used for the development of metabolic and cellular engineering strategies. Chips and microarrays are becoming standard tools for the high-throughput analysis at the level of gene expression. Chip systems also enable the rapid characterization of the desired recombinant product even in solutions from process intermediates (Forrer *et al*., 2004, Vasilyeva *et al*., 2004).

Analyzing the transcriptome profiles by DNA microarrays shows that the growth phase can significantly affect the transcriptome profiles of *E. coli* during well-controlled synchronized high-cell-density fed-batch cultures (Haddadian and Harcum, 2005). Hermann (2004) analyzed transcriptome profiles of recombinant *E. coli* producing the human insulin-like growth factor I fusion protein during HCDC fed-batch culture using DNA microarrays. The expression levels of 529 genes were significantly altered after induction. About 200 genes were significantly downregulated during the production of protein after induction. Physiological and metabolic changes of *E. coli* observed by proteome analysis via gel electrophoresis (2-DE) are summarized as follows: The levels of TCA cycle enzymes (isocitrate dehydrogenase, malate dehydrogenase, succinate dehydrogenase and succinyl-CoA synthetase) increased during the exponential phase of HCDC, while the levels of glycolytic enzymes, (enolase, fructose-bisphosphate aldolase, phosphoglycerate mutase 1, triose-phosphate isomerase) decreased during the stationary phase. (Hermann 2004). The synthesis of isocitrate dehydrogenase increased considerably (up to four-fold) in the exponential growth phase. On the other hand, levels of most amino acid biosynthetic enzymes decreased during this phase of growth.

Raman *et al*. (2005) used proteome analysis to evaluate the differences in protein expression of recombinant *E. coli* in glucose limited fed-batch fermentation. The authors reported that gene up-regulation in glucose limited fed-batch cultures equips cells for the scavenging of glucose (which is present at low concentrations), transporting and metabolizing of a wide range of substrates, tackling energy deficiency and coping with stressful conditions. Yoon *et al*. (2003) used combined transcriptome and proteome analysis during high cell density fed batch culture of *E. coli* in order to understand physiological and metabolic changes during HCDC. The authors reported that the expression of genes involved in translation, ATP synthesis and amino acid synthesis was downregulated after feeding but expression of most genes of the TCA cycle and genes which are involved in overcoming undesirable intracellular conditions was upregulated. Another interesting phenomenon observed by proteome profiling was the change in the permeability of the outer membrane as cell density increased. The expression of chaperone genes increased with cell density, which is an inevitable consequence of the stress imposed on the cell at high cell densities, which may also turn out to be beneficial for the production of correctly formed heterologous proteins (Makrides, 1996).

The use of these pioneering analyses is not limited to *E. coli*, although high cell density cultures of other microorganisms have rarely been studied. Examples concerning the use of high throughput analyses for other microorganisms like: *Lactococcus lactis* (Vido *et al*., 2004), *B. subtilis* (Helmann *et al*., 2003), *Corynebacterium glutamicum* (Ruckert *et al*., 2003), *Aspergillus terreus* (Askenazi *et al*., 2003), *S. cerevisiae* (Salusjarvi *et al*., 2003) and *P. putida* (Heim *et al*., 2003) can be found in literature.

These findings should be invaluable in designing metabolic pathways and fermentation strategies for the production of recombinant proteins and metabolites by HCDC of *E. coli*. Unfortunately, there is little information on the transcriptome and proteome of other microorganisms.

Another problem associated with HCDC is filamentation which is a response to the high density of cells. Filamentation of cells lowers the final achievable cell concentration and the productivity of the target protein. The expression of foreign proteins enhances the biosynthesis of the repressor of the cell division proteins FtsZ and FtsA and has been found to hamper the productivity. Over-expression of FtsZ or FtsA allows unconditional cell division and consequently, high density growth and high productivity (Jeong and Lee, 2003; Wang and Lee, 1998; Lee, 1994).

Table 3. Methods used to deal with acetate accumulation in recombinant *E. coli*.

Method	Process description	Reference
Using different expression systems	*E. coli* B (which produces less acetate) were used instead of *E. coli* K	Noronha *et al*. (2000); Phue *et al*. (2005)
Mutants with defects in acetate biosynthetic pathway	Mutants of *E.coli* w3100 were generated which lacked acetate associated enzymatic activity and produced less acetate	Contiero *et al*. (2000)
Enhancing acetate utilization	Mutants were generated which could consume acetate as carbon source even in the presence of glucose	Oh *et al*. (2002)
Converting acetate to other (non-toxic) by-products	Acetate was converted to other by-products (e.g. acetone or acetoine) which are not toxic for the cell	Bermejo *et al*. (1998) Aristidou *et al*. (1995)
Blocking the pathway of by-product production	Antisense RNA was used to partially block the production of toxic by-products without affecting other vital processes of the cell	Kim and Cha (2003)
Redirecting the metabolic fluxes	Carbon flux was redirected through phosphor-phenol puyruvate and glyoxylate shunt and production of acetate was minimized	Farmer and Liao (1997)

3. Culture condition improvement: In order to develop an optimized condition in terms of medium composition and physical conditions for reaching higher productivity via higher cell density and/or specific productivity, there are some points which should be considered:

3.1. Medium composition

It is desirable to make the feed solution as simple as possible by including the essential non-carbon, non-nitrogen components in the medium. But it should be borne in mind that some nutrients can inhibit cell growth when present above a certain concentration (Lee, 1996). High amounts of substrates are needed to achieve high cell density but these substrates should be fed in a controlled manner because they may have adverse effects on cell growth and production. Excess carbon source leads to metabolic by-products which are inhibitory and can be prevented by feeding a limited supply of carbon source. The main metabolic by-products are acetate for *E. coli*, propionate for *B. subtilis*, lactate for *L. lactis* and ethanol for *S. cerevisiae* (Riesenberg and Guthke, 1999).

Another point is the precipitation of media ingredients, especially when they are present at high concentrations, which is usually the case when the cells are to be grown to high densities (Shiloach and Fass, 2005). Precipitation can affect downstream recovery, purification operations and monitoring devices. For example precipitation of mineral salts which may occur during medium preparation hampers the determination of the actual concentration of minerals in the medium; it can also complicate the measurement of cell densities (Cereghino *et al*., 2002). Seeking a solution to the above mentioned problems, Brady *et al*. (2001) cut the concentration of all salts in the medium to one quarter of the original recipe. Another concern is the osmotic pressure and conductivity caused by high ion concentrations in the growth media that may affect membrane potential and activate different stress mechanisms that induce reduction in growth rate or termination of the growth cycle (Winzer *et al*., 2002). Generally, defined media are used to obtain high cell density because the nutrient concentrations are known and can be controlled during culture (van Hoek *et al*., 2000). Complex media such as peptone and yeast extract can vary in composition and quality making fermentation less reproducible. However, semi-defined or complex media are sometimes necessary to boost product formation. The use of a defined medium with a single or a few amino-acids to achieve higher cell or/and recombinant protein yields would be attractive for industrial conditions. It has been reported that adding a dose of leucine at the beginning of an *E. coli* culture with continuous feeding of glucose, threonine, tryptophan, and histidine improved productivity of β-isopropylmalate dehydrogenase (Rozkov *et al*., 2001). Li *et al*. (1998) reported that the addition of precursor amino acids (glutamate, cysteine and glycine) and ATP improved intracellular glutathione accumulation in HCDC of *E. coli* Addition of certain amino acids has also been shown to be fruitful in yeasts such as *S. cerevisiae*. Gorgens *et al*. (2005) supplemented the medium with a balanced mixture of alanine, arginine, aspargine, glutamic acid, glutamine and glycine to enhance heterologous protein production in a defined medium, such an approach has also been shown to be useful in another study (Jin and Shimizu, 1997). But, it is worth mentioning that sometimes the addition of amino acids which are present in the biomass and recombinant protein in similar amounts may even decrease the yield. For example, increasing concentration of phenylalanine resulted in a lower chloramphnicoleacetyl transferase (CAT) concentration, presumably due to feedback inhibition of biosynthesis of this amino acid and sharing common biosynthetic pathways (Ramirez and Bentley, 1993). Lee *et al*. (2000) applied phosphorus limitation during fed-batch culture by reducing the initial KH_2PO_4 concentration in order to increase the polyhydroxy alkanoate concentration. Cell density of *P. putida* also increased with this modification to 141 g/l. Lau *et al*. (2004) increased the maximum cell density by two-fold, and the final titer of product (6-deoxyerythronolide B) by 11-fold by doubling the concentration of phosphate and continuous feeding of propionate and maintaining a low propionate concentration (5-10 mM) in the medium.

For fed-batch process, which is the most common strategy for HCDC, it is desirable to simplify the feed solution as much as possible by including sufficient non-carbon and non-nitrogen nutrients in the starting medium (Lee, 1996). However, different studies report that the addition of some materials to the feeding solution can significantly improve the productivity. Oh *et al*. (2002) controlled the density *of B. subtilis* by controlling the ratio of glucose and peptone concentrations in the feeding medium. Jeong *et al*. (2004) investigated chemically defined-, yeast extract-containing, and casamino acid-containing-feeding solutions for the production of human leptin by fed-batch culture of recombinant *E. coli*. Among these solutions, casamino acids led to the highest productivity.

In short, new medium optimizations are necessary for the production of new recombinant proteins which seem to differ with respect to the type of microorganism and the product. It appears that enhancing amino acids and other compositions are still a good choice which have been used by many researchers. The basic approaches used to develop optimal media were trial-and-error processes. However, the use of statistical techniques for experimental design has provided a more elegant means of designing.

3.2. Physical conditions

Temperature: For high cell density cultures, temperature control is much more important due to significant heat release in spite of limited heat transfer because of high viscosity. Temperature should support cell growth as well as product formation. Since in most fermentation processes, growth phase is separated from production phase, temperature should be optimized for each phase while maintaining nutrient characteristics. It has been reported that temperature affects plasmid stability and consequently the yield of protein production in culture (Donovan *et al*., 1996). It has been demonstrated that the rate of mRNA degradation is a first order reaction and decreases with temperature. Thus it is possible that lowering culture temperature could be a simple and a potentially important method for increasing protein production (Shin *et al*., 1997).

Oxygen: In high cell density cultivation, a high capacity of oxygen supply is required. Oxygen often becomes limiting in HCDCs owing to its low solubility. The saturated dissolved oxygen (DO) concentration in water at 25ºC and 1 atm is ~7 mg/l, but oxygen supply can be increased by increasing the aeration rate or agitation speed (Lee, 1996). Oxygen-enriched air or pure oxygen has also been used to prevent oxygen limitation. Cells can also be cultured under pressurized conditions to increase oxygen transfer (Belo and Mota, 1998; Lee, 1996). By increasing oxygen transfer capacity of the bioreactor, it is possible to achieve higher cell productivity and final biomass concentration; because oxygen limitation results in formation of several metabolites of the mixed acid metabolism such as succinate, acetate, lactate, ethanol, and hydrogen which are undesirable and decrease the productivity of the bioreactor. (Castan *et al*., 2002; Enfors *et al*., 2001). However, when oxygen enriched air or pure oxygen is used to achieve high feed rate, the impact of high oxygen concentrations on the productivity and quality of recombinant proteins production needs to be investigated. Also it should be considered that oxygen itself is potentially toxic to some microorganisms.

Carbon dioxide: Carbon dioxide can also affect cell growth and recombinant protein production especially in high cell densities (Lee, 1996). High feed rate of the limiting substrate results in high carbon dioxide production rates and thus a high carbon dioxide concentration in the bioreactor. The dissolved carbon dioxide concentration depends on the partial pressure of the carbon dioxide according to Henry's law. Growth inhibition and toxic effects of carbon dioxide have been reported (Castan *et al*., 2002). High partial pressure of CO_2 (>0.3 atm) decreases growth rate and stimulates acetate formation (Lee, 1996). Therefore, the pressurized culture regime which has been used to increase oxygen transfer may also enhance the detrimental effect of CO_2 (Matsui *et al*., 2006).

Mixing: Reduced mixing efficiency of the bioreactor is another physical limitation of HCDC due to high viscosity. This problem intensifies with increasing bioreactor size (Lee, 1996). In large scale bioreactors there are fluctuations in the concentration of the limiting substrate due to difficulties in mixing. In these processes, zones of high and low substrate concentrations are formed. In high concentration zones cells may produce toxic by-products and are prone to oxygen limitation but in low concentration zones cells may be starved of substrate. Another problem associated with this situation is that cells also have to face an imposed stress because of continuously passing through zones of high and low substrate concentrations. Increasing the rate of agitation is the main solution of these problems, this method can enhance protein formation and the volumetric oxygen transfer coefficient (Zhang *et al*., 2005; Kapat *et al*., 1998) but it may have detrimental effects on cells which are sensitive to shear stress like animal cells (Pan *et al*., 2000). Considering these disadvantages feeding in several points in the reactor and reducing the concentration of the feed have been proposed as possible solutions (Enfors *et al*. 2001).

Foaming: Foam formation may cause serious operational difficulties in aerated stirred bioreactors, especially in high cell density cultivation for recombinant protein production. Because with increasing cell density, cell lysis and consequently, protein concentration in the medium increases thus enhancing foam formation. Various procedures have been used in industry to reduce foam formation rate, with each of them having its own advantages and disadvantages. Stirring as foam disruption (SAFD) technique is a novel method to reduce foam in fermentation processes. The principle of this method is to reduce the foam layer with liquid flow generated by a stirrer placed just under the gas-liquid interface (Hoeks *et al*., 2003).

4. Growth technique improvement: Method of cultivation is important to the success of high cell density and recombinant protein production, because it affects environmental and nutritional conditions that are effective in microorganism's growth and recombinant protein production. For this reason different methods, focusing on nutrient feeding strategies, have been developed to grow cells to high cell densities and to overproduce protein. The most important function of every strategy is to prevent overfeeding in which inhibitory concentrations of the feed components accumulate in the fermentor, or underfeeding in which the organism is starved for essential nutrients. The method of choice depends on many different factors, including the metabolism of the organism, the potential for production of inhibitory substrates, induction conditions and the capacity to measure parameters. Batch (Castrillo *et al*., 1996), continuous (Domingues *et al*., 2005 and 2000), semi-continuous (Elias *et al*., 2000), continuous with recycling (Tashiro *et al*., 2005) and a variety of fed-batch processes (see below for examples) have been reported for growing cells to high densities. Fed-batch is the most commonly used method to produce recombinant proteins by HCDCs.

4.1. Fed-batch processes

The fed-batch process is a suitable strategy for production in high cell density culture due to (1) extension of working time (particularly important in the production of growth-associated products), (2) controlled conditions for the provision of substrates during fermentation and (3) control over the production of by-products, or catabolite repression effects, due to limited provision of only those substrates which are solely required for product formation.

In fed-batch cultivation, feeding strategy is the most important factor in success of the process. Different feeding strategies including constant-rate feeding, stepwise increase of the feeding rate, and exponential feeding have been used to obtain high cell densities in fed-batch cultures (Shiloach and Fass, 2005; Lee, 1996). In constant-rate feeding, concentrated nutrients are fed into the bioreactor at a predetermined rate. Because of the increase in culture volume and cell concentration in the bioreactor, the specific growth rate continuously decreases, and the increase in cell concentration slows down over time (Jensen and Carlsen, 1990). Variable feeding rates can be controlled with feedback or without feedback. The stepwise (or gradual) increase of the feeding rate can enhance cell growth by supplying more nutrients at higher cell concentrations (Jensen and Carlsen, 1990; Konstantinov *et al*., 1990). Cells can grow exponentially during the entire culture period if the feed rate of the growth-limiting substrate is increased in proportion to growth (Shiloach and Fass, 2005; Yee and Blanch, 1993; Strandberg and Enfors, 1991). The exponential-feeding method has been developed to allow cells to grow at constant or variable specific growth rates; it also provides the advantage that acetate production, a serious problem associated with the process, can be minimized by controlling the specific growth rate below the critical value of acetate formation (Table 3). Exponential feeding is a simple but efficient method that has been successfully used for high cell density cultivation of several non-recombinant and recombinant microorganisms; the specific growth rate is usually maintained between attainable maximum and minimum values. Maintaining the specific growth rate at an appropriate range can provide a desirable metabolic condition and results in maximum productivity (Babaeipour *et al*., 2007). Therefore, exponential feeding can be used as a convenient method to avoid by-product formation and to obtain maximum attainable cell density (Shiloach and Fass, 2005; Khalilzadeh *et al*., 2004 and 2003; Tabandeh *et al*., 2004; Thuesen *et al*., 2003; Lee, 1996; Yee and Blanch, 1993) but, the details of such feeding are still a matter of debate and new researches aim at optimizing the feeding method (Babaeipour *et al*., 2008; Bahrami *et al*., 2008; Ting *et al*., 2008).

In addition to conventional fed-batch processes, there are some modified fed-batch cultivation techniques, mentioned below, which use special strategies to control the process.

4.2. Two stage, cyclic fed-batch process

Two stages, cyclic fed-batch process is a modified fed-batch process that entails transfer of a portion of the whole fermentation broth from the growth stage to the production stage while leaving a smaller fraction of the broth for continued cell growth in the growth stage. The volume of broth in the growth stage can then be replenished to its pre-transfer volume at a predetermined optimal rate while induction of gene expression and production are taking place in the production stage. The optimal process conditions in the production stage, such as pH, temperature, cell growth rate and medium composition can be controlled and maintained independently from the optimal conditions in the growth stage (Chang *et al*. 1998; Curless *et al*. 1991). Chang *et al*. (1998) obtained a two fold increase in volumetric productivity of rice α-amylase productivity by the yeast *Yarrowia lipolytica SMY2* in comparision with a conventional fed-batch process. Choi *et al*. (2001) used a two-stage fed-batch process for the production of human granulocyte-colony stimulating factor. They optimized the pre-induction growth rate and the feeding strategy during the production stage. Genetic stability of the recombinant strain and the design of optimal media for growth and production stages are also critically important to a successful implementation of the two-stage, cyclic fed-batch process for production of heterologous protein and when working in large scale. Thus the risk of contamination and economical concerns will also become an issue.

4.3. Temperature-limited fed-batch (TLFB) process

The temperature-limited fed-batch process is a technique where the oxygen consumption rate is controlled by a gradually declining temperature profile rather than a growth-limiting glucose-feeding profile. Two mechanisms that may contribute to the much higher accumulation of product in the TLFB process are: 1) reduced proteolysis due to lower temperature, 2) reduced proteolysis due to lower cell death and protease release to the medium (Jahic *et al*., 2003).

In *E. coli* cultures, this method has been proved to prevent an extensive release of endotoxins, i.e. lipopolysaccharides, which occur in glucose-limited fed-batch processes at specific growth rates below 0.1 h^{-1} (Svensson *et al*. 2005; Han and Zhong, 2003). This technique stabilizes the cell membrane towards osmotic shock which results in reduced contamination of the considered periplasmic protein extract with cytoplasmic proteins and DNA (Svensson *et al*., 2005).

Mare *et al*. (2005) used a cultivation strategy combining the advantages of temperature-limited fed-batch and probing feeding control. The temperature was decreased to lower the O_2 demand and the growth rate. A mid-ranging controller structure was used to manipulate the temperature and the stirrer speed to control the dissolved O_2 tension. The probing feeding strategy is changed when the maximum stirrer speed is reached to obtain a slight excess of glucose. The resulting strategy thus limits the growth rate without the risk of acetate accumulation. A 20% increase in cell mass was achieved and the usual decrease in specific enzyme activity normally observed during the late production phase diminished with the new technique.

4.4. A-stat

The A-stat technique is a combination of continuous and fed-batch techniques (Paalme *et al*., 1995; Paalme and Vilu, 1992). It is basically a continuous culture with a smooth change of the desired growth rate. At first, like in a chemostat, a steady-state culture is obtained. After that, the computer controlled smooth change of dilution rate, while keeping its time derivative constant, is started and continued up to almost complete washout. This technique showed to be a powerful technique for the quantitative study of cell physiology, being at the same time considerably less time consuming and more informative than the conventional chemostat. Also, cultures seem to react better to a smooth rather than an abrupt change in the dilution rate (Paalme *et al*., 1997; Paalme *et al*., 1995). However, the system is more suitable for academic purposes and no reports about using this system in industry have been reported to date.

4.5. Dialysis fermentation

Dialysis fermentation is a way to overcome the inhibitory effect of acetate and other nutrients and to obtain high cell density growth. Dialysis is defined as the separation of solute molecules by their unequal diffusion through a semi-permeable membrane based on a concentration gradient. Two configurations of vessel arrangement as mentioned by Shiloach and Fass (2005) were proposed for dialysis reactors: 1) two-vessel reactor consisting of a culture reactor that had a medium reservoir connected by a dialysis device; 2) a single-vessel dialysis reactor consisting of two chambers separated by a dialysis membrane. The single vessel arrangement is less preferable because it is difficult to sterilize and sensitive to mechanical stress and oxygen limitation (Fuchs *et al*., 2002; Markl *et al*., 1993). The highest cell density recorded by membrane dialysis reactors is 190 g/l for *E. coli* (Nakano *et al*., 1997). Because of successful high cell density cultivations of *E. coli* in a laboratory dialysis reactor, a scale-up of the process was investigated by Fuchs *et al*. (2002). Seeking to provide sufficient membrane area for dialysis in a technical scale fermentor, they used an external membrane module, which was also applied for oxygen supply to the culture in the external loop. Cell densities exceeding 190 g/l, previously obtained in laboratory dialysis fermentation, were also produced with external dialysis modules. Protein concentration in a 300-L reactor was increased to 3.8-fold of industrial fed-batch-fermentations. However, despite the promising results obtained in this study, no further reports about the academic or industrial usage of this technique for HCDC have been reported to date.

4.6. Pressurized cultivation

Matsui *et al*. (2006) showed that an air-pressurized culture is able to meet the high demand for oxygen in the HCDC of *E. coli* Carbon dioxide generated by the cells under increased pressure was inhibitory and as a result, cellular growth stopped in the air-pressurized culture at a constant mass flow rate. Increasing the flow rate along with the pressure in the reactor enabled the *E. coli* cells to grow to 130 (non-recombinant) and 104 (recombinant) g/l due to the release of the CO_2. In addition, the specific activity of the product, tryptophan synthase, was increased.

4.7. Perfusion techniques

The basic characteristics of perfusion systems are constant medium flow, cell retention and in some cases, selective removal of dead cells. Cell retention is usually achieved by membranes or screens, or by a centrifuge capable of selective cell removal. Perfusion systems are most often used for animal cell culture. Advantages and disadvantages of using this technique are shown in Table 4.

Kiy *et al*. (1996) by continuous exchange (at an optimized perfusion rate) of the medium, after an initial batch phase, obtained cell densities and enzyme activity, 20 and 50 times, respectively higher than standard batch fermentations of *Tetrahymena thermophila*. Scheidgen-Kleyboldt *et al*. (2003) applied

Table 4. Advantages and disadvantages of using the perfusion technique

Advantages	Disadvantages
Removal of cell debris and inhibitory byproducts	Large amounts of medium are needed
Removal of enzymes produced by dead cells	Nutrients are less completely utilized than in batch and fed-batch cultivation
Shorter exposure of product to harsh operational conditions (pH or temperature)	Increased cost of waste treatment
High volumetric productivity	-

the same strategy for producing hydrolytic enzymes by continuous high cell density cultivation of *Colpidium campylum*. Yang *et al*. (2000) increased the volumetric antibody productivity by using a "controlled-fed perfusion" approach, nearly twofold over the perfusion process, and surpassed fed-batch and batch processes by almost tenfold. The substantial boost in the overall productivity is attributable primarily to the combined effects of increased cell density as well as reduced product dilution. Perfusion techniques seem to be a very good choice especially for the production of recombinant proteins from plant cell cultures. However, it seems that investigations should still be carried out to optimize bleeding rates and study cell physiology in perfusion cultures (Su and Arias, 2003).

5. Induction condition: As previously mentioned, over expression of a protein places an additional metabolic burden on the cell's energy and carbon and amino acid pools, which may result in reduced cell growth. This can be avoided by employing inducible expression systems. Of course, induction of recombinant protein production results in a great change to the transcriptome. The major difference between the induced recombinant cultures and the non-induced wild-type cultures is the significant down-regulation of the gene families responsible for protein production, i.e. energy synthesis, transcription, and translation genes (Haddadian and Harcum, 2005). The inducer can be a chemical or change of a physical parameter such as temperature. The amount of inducer, the strategy of its addition and culture conditions in time of induction can affect the efficiency of induction. The optimum induction strategy can be determined by trial-and-error methods or taking the effects of various cultivation conditions on the recombinant gene expression into account (Shin *et al*., 1997).

5.1. Quality of inducer

Many inducible promoters have been developed, which can be induced by various mechanisms such as temperature shifting, pH change and addition of chemical inducers. An overview of inducible promoters for HCDC has been shown in Table 5.

Considering the advantages and disadvantages of using different promoters, it can be concluded that *lac* based promoters are still the first choice to be used in HCDC. But, there is a chance that in the near future lactose can replace IPTG as the inducer as it is less expensive and can be used as an additional carbon source. Other promoters, although less expensive than *lac* based ones, still have many disadvantages. Should the researchers or the industry want to use these promoters, there are still lots of improvements that should be done to overcome these disadvantages.

5.2. Quantity of inducer

The amount of inducer required to titrate the repressor molecules is proportional to the total cell mass and the optimal specific concentration of the inducer, therefore it needs to be determined for maximizing the recombinant protein synthesis at any cell concentration. The level of inducer required for optimal expression depends on the strength of the promoter, the presence or absence of repressor genes on a plasmid, the cellular location of the product, the response of the cell to recombinant protein expression, and the solubility of the target protein and the characteristics of the protein itself (Cserjan-Puschmann *et al*., 2002; Donovan *et al*., 1996).

For example, Shin *et al*. (1997) tested a range of specific amounts of inducer (IPTG) (3.26×10^{-3} to 5.11×10^{-2} mmol/g of cell) on production of mini-proinsulin and reported 5.11×10^{-2} mmol/g of cells as optimum concentration. Vidal *et al*. (2005) investigated the

Table 5. Inducible promoters which are usually used in HCDC.

Promoter	Example	Inducers	Advantages	Disadvantages
T7 or *lac*-based promoters	*tac, trc, lac, lacUV5-T7* hybrid	Isopropyl-β-D-thio-galactopyranoside (IPTG)	Products are effectively induced	Toxicity and high costs of IPTG, Difficult to use in large scale
		Lactose	Less expensive and toxic than IPTG, can be used as extra carbon source	Difficult to use in large scale
Positively regulated systems	arabinose-inducible *PBAD* promoter, Rhamnose-inducible *rha*BAD promoter	----	----	Product quality decreases as cell density increases
Starvation-induced promoters	*Trp, phoA*	Exhaustion of a specific substrate	----	Substrate exhaustion interferes with production, time of induction is not known
Heat-inducible promoters	λP_L	Temperature shift	----	Temperature shift adversely affects production, difficult to use in large scale

influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by *E. coli* using the T5 promoter. They reported that working in fed-batch, batch and shake flask cultures at the same IPTG concentration gives the same level of specific activity. They also reported that growth and enzyme production rates are reduced by increasing the IPTG concentration in batch and fed-batch strategies up to the range of 200 to 1500 μmol IPTG/l.

In general, for inducing the expression of an intracellular recombinant protein, the use of 1mmol IPTG/l is a reasonable starting point because maximal induction is predicted to occur for both lacI and lacI^q at this level (Laffend and Shuler, 1994). For secreted proteins however, IPTG concentrations of 0.01 to 0.1 mmol/l is suitable to minimize potential problems due to product insolubility, growth inhibition and cell lysis (Lee and Ramirez, 1992).

5.3. Induction time

The other important parameter for the development of the optimized induction strategy is induction time, because maximum yield of foreign proteins in fermentation depends on the point in the growth cycle at which expression is induced. For strains whose growth and/or viability are drastically reduced following induction, induction in late-logarithmic or stationary phase provides high cell densities for increased product formation. However, as shown for chloramphenicol acetyl transferase (CAT) expression under the control of the *tac* promoter (Donovan *et al*., 1996), low growth rates and protease activity brought on by depleted nutrient levels in the stationary phase can reduce the yield of foreign protein. In this case, optimal induction in the mid-logarithmic phase provided sufficient levels of CAT protein within the cell while achieving a high cell density to produce the maximal yield. When product expression is low and/or does not significantly influence cell growth, overall foreign protein yield will be maximized by inducing expression throughout the entire growth phase (Donovan *et al*., 1996).

Tuning the expression of recombinant gene in relation to the metabolic capacity of the host cell synthesis machinery to extend the production phase and to attain maximal yield is a new suitable strategy for increasing productivity and yield of recombinant protein. In this regard, a novel concept of transcription rate control by continuous supply of limiting amounts of inducer in a constant ratio to biomass was developed and implemented in process with a carbon limited exponential feed regime of medium and inducer (Striedner *et al*., 2003; Cserjan-Puschmann *et al*., 2002; Grabherr *et al*., 2002). Although, increasing the duration of the induction phase enhances the release of periplasmic proteins to the surrounding environment (Mergulhao *et al*., 2005). Gombert and Kilikian (1998) investigated adequate induction strategies for adding lactose as inducer to the bioreactor by testing the number of pulses and time intervals between two consecutive pulses. The time when glucose is nearly depleted may be an optimal time for inducing recombinant protein expression with lactose (Donovan *et al*., 1996; Neubauer *et al*., 1992). This may be because of the induction of starvation responses, which results in a longer production phase of the recombinant product (Lin *et al*., 2004).

5.4. Medium condition at induction phase

Temperature and composition of growth medium during induction can significantly affect foreign protein expression. Inducer (s) can also be used as carbon or nitrogen source. Resina *et al*. (2005) applied methylamine and sorbitol as nitrogen and carbon sources, respectively for the induction phase of recombinant lipase production in a high cell density culture of *Pichia pastoris*. Furthermore, according to cells' need some materials may be added during the induction phase to improve foreign protein expression. It has been shown that providing additional amino acids by supplementing the medium with casamino acids, peptone or yeast extract during induction leads to an increase in productivity (Madurawe *et al*., 2000; Gombert and Kilikian, 1998; Nancib *et al*., 1991; Li *et al*., 1990) and stability (Whitney *et al*., 1989). For example, supplementing the medium with particular amino acids based on the amino acid sequence of recombinant interferon-γ significantly increases the productivity (Khalilzadeh *et al*., 2003).

Induction temperature can also affect productivity. Decreasing induction temperature may enhance functional protein formation by reducing the rate at which an over-expressed protein is formed. Reduced expression rates reduce the concentration of unfolded (recombinant) intermediates in the cell. However, at a case study it has been reported that with lowering induction temperature from 37 to 30ºC recombinant proinsulin production decreased considerably during fed-batch cultivation of *E. coli* (Shin *et al*., 1997). Therefore, decreasing induction temperature is not a general rule for increasing production and optimization of induction temperature is necessary for all expression systems.

6. Process analysis and control

Analytical controls ensure a consistent performance of the defined process while makeing it possible to evaluate the effect of applied changes to the process on productivity before and after implementation of process changes (Graumann and Premstaller, 2006). As Shimizu *et al*. (1993) pointed out, the control-system development for biological systems is not straightforward due to (1) the lack of accurate models describing cell growth and product formation, (2) the nonlinear nature of the bioprocess, (3) the slow process response, and (4) a deficiency of reliable on-line sensors for the quantification of key state variables, several attempts have been done to analyze and control HCDC.

Several variables are being used for control purposes and can be classified (Lee *et al*., 1999a) as either measured or manipulated. Measured variables can be classified further as either directly measured (on-line or off-line) or indirectly determined. Directly measured variables include temperature (T), pH, dissolved oxygen concentration (DO), optical density (OD), substrate concentration (s), pressure and exit gas composition. These variables can be measured directly during cultivation by various instruments such as DO probes, pH probes (pH), T probes (T), spectrophotometers (OD), high-performance liquid chromatography (*s*), glucose analyzers, gas chromatographs and mass spectrometers. Indirectly determined variables include specific growth rate (μ), cell concentration (*x*), oxygen uptake rate (OUR), oxygen transfer rate (OTR), carbon dioxide evolution rate (CER), glucose (or other substrates) uptake rate (GUR), glucose (or other substrates) demand (GD) and respiratory quotient (RQ). Indirect variables are estimated or calculated from one or more of the directly measured ones. The manipulated variables include agitation speed and substrate feed rate. Most of these variables have been used in combination to determine the nutrient feed, usually the most critical factor in high cell density processes. For evaluating the quality of a measurement, calibration/checking prior to and after cultivation by mounting identical sensors in well comparable positions and checking the individual signals for quality and elemental balancing often for carbon and nitrogen is usually carried out (Galvanauskas *et al*., 1997; Chattaway *et al*., 1992; Shuler and Kargi, 1992).

The analytical method should be easy-to-use, quick and reproducible while maintaining an adequate information content. Graumann and Premstaller (2006) reviewed a number of new analytical systems that have recently been introduced to the field of biotechnological production of recombinant proteins which increases the flexibility and sophistication of feed control schemes available for HCDC process.

The new advances such as chemometric sensors (Clementschitsch *et al*., 2005), optical sensors (Marose *et al*., 1999) and other on-line or off-line measurements of product, nutrients and metabolites (for examples see Meuwly *et al*., 2006; Crowley *et al*., 2005; Bélanger *et al*., 2004; Peuker *et al*., 2004; Baker *et al*., 2002; Rocha and Ferreira, 2002; Hoffmann *et al*., 2000) contribute to close gaps remaining in the understanding and control of HCDC process. In spite of all the researches mentioned above, widespread usage of new analytical systems has been hampered by several problems including poor thermal stability (e.g. enzyme electrodes), poor reliability or a high level of complexity (e.g. filtration type systems and flow injection analysis (FIA) systems) (Lee *et al*., 1999).

As previously mentioned, usually the most critical factor is nutrient feeding which should support cell growth and recombinant protein production while avoiding substrate inhibition and other related problems. The simplest control is open-loop control, which means controlling without feedback. Open-loop controls can be applied for constant-rate feeding, gradual stepwise or linear increase of the feeding rate and exponential feeding based on fermentation model equations derived from mass balances (Lee, 1996, Shiloach and Fass, 2005). Combination of these trends is also possible. In feedback control (close-loop), a measured variable and a manipulated variable will be considered to be controlling the process. In direct feedback control, the measured variable and the manipulated variable are the same, but usually these are different (indirect feedback control) and the measured variable can be used directly to adjust manipulated variable or can be used for estimating a variable that will be used to set a manipulated variable.

On-line analyzing of substrate is an example of direct feedback control in fed-batch processes. The concentration of carbon source in the culture medium can be controlled at a desired value if we can measure it on-line (Lee *et al*., 1999). As an example, Kim *et al*. (1994) used a glucose analyzer for fed-batch culture of *Alcaligenes eutrophus* for the production of poly (3-hydroxybutyrate). They clearly showed that controlling nutrient concentration in an optimal range is an efficient way of cultivating cells to high concentration, even though this is a simple single-input/single-output (SISO) system. Kellerhals *et al*. (1999) developed a closed-loop control system based on on-line gas chromatography for assaying Na-octanoate, as the sole carbon source, to maintain continuously fed substrates at desired levels. In another study, Shang *et al*. (2006) controlled glucose feeding rate in accordance with ethanol concentration which is the by-product of the process of ergosterol production in high cell density cultivation of *S. cerevisiae*. Due to the delay in measurement and instability of on-line glucose systems, methods that estimate and predict substrate consumption rate are generally preferred (Lee *et al*., 1999). Meuwly *et al*. (2006) illustrated that glucose consumption rate (GCR) can be successfully applied as an indirect method to monitor and control high-density perfusion cultures of Chinese hamster ovary cells in packed-bed bioreactors.

Other direct feedback control strategies such as DO, pH, cell concentration and exit gas composition have been applied to control the process. The DO-stat method is based on the finding that the DO increases sharply when the substrate is depleted. Therefore, the substrate concentration can be maintained within a desired range of nutrient when the DO rises above the preset value (Lee, 1996). Konstantinov *et al*. (1990) introduced the balanced-DO-stat method which guaranties sufficient oxygen supply and prevents overfeeding. They measured the exit gas composition from the fermentor in real time, estimated the GUR and determined the nutrient (or glucose) feed rate. Akesson *et al*., (2001) avoided acetate accumulation in HCHC by feedback controlling of glucose feeding based on oxygen probing. Whiffin *et al*. (2004) developed a starvation-based dissolved oxygen (DO) transient controller to supply growth limiting substrate to high cell density fed-batch cultures of recombinant *E. coli*. The algorithm adjusted a preexisting feed rate in proportion to the culture's oxygen demand, which was estimated from fluctuations in DO concentration.

The pH-stat method is based on the observation that the pH changes when the primary carbon substrate becomes depleted or abundant (Kim *et al*., 2004; Choi and Lee, 1999a,b; Lee and Chang, 1993). When the carbon substrate in the culture is exhausted, pH begins to rise mainly as a result of catabolizing organic acids or amino acids as carbon or energy sources. Shin *et al*. (1997) increased the volumetric media feed rate in a stepwise manner during the feeding-on period as the cell concentration increased during the pH-stat production of mini-proinsulin with *E. coli*. Kim *et al*. (2004) used this control strategy to grow recombinant

E. coli up to 101 g/l by controlling the specific growth rate at 0.11/h, when pH rised above an upper limit due to the depletion of substrate, feeding got started.

In a defined medium, the DO-stat responds more rapidly to nutrient depletion than the pH-stat. But, when complex carbon-nitrogen substrates such as yeast extract or peptone are used together with carbohydrate substrates, the DO change is not as large as when the carbon source is depleted, since the cells utilize the complex substrates (Lee, 1996). Therefore, the pH-stat method is more suitable when semi-defined or complex media are used.

Cell concentration can also be used for indirect feedback control if suitable detectors such as a laser turbidimeter for on-line analyzing of the cell concentration exist. Exit gas compositions are measured to estimate specific state variables, namely OUR, CER, RQ, GUR and the ratio of OUR to GUR (Lee *et al*., 1999). For example, cells produce CO_2 during growth and the CER is roughly proportional to the carbon source consumption rate. Therefore, nutrient feeding can be controlled by using CER data that can be calculated from the concentration of CO_2 in the gas outlet (lee, 1996).

Chung *et al*. (2006) reviewed robust adaptive controllers and expert systems based on fuzzy control or neural networks and introduced a new multiple-model control strategy for fed-batch high cell-density culture processing.

7. Concluding remarks and future prospects: As discussed in this review, several approaches at different levels are available for increasing productivity in high cell density cultures. Information on genome, transcriptome and proteome levels is a great help for genetic engineers and biochemists to design and construct a well-adapted host for HCDCs. Designing a suitable medium as well as nutrient strategy for supporting growth and the production phase is another concern for biotechnologists. Optimizing physical conditions for enhancing mass and heat transfer and decreasing foam formation is an obstacle for chemical engineers. Although, the effects of high cell density on *E. coli* metabolism has been studied, further investigations should be focused on understanding the global cellular response of *E. coli* and other microorganisms to harsh conditions especially related to recombinant protein production in high cell density cultures.

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