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 Babaeipour¹, Amir Mohammad Farnoud¹

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

Table of Contents:

- 1. Introduction
- 2. Expression system improvement
- 3. Culture condition improvement 3.1. Medium composition 3.2. Phusical conditions
- 4. Growth techniques imporvements 4.1. Fed-batch processes

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

- 4.2. Two stage cyclic fed-batch process
- 4.3. Temperature-limited fed-batch (TLFB)
- 4.4. A-stat
- 4.5. Dialysis fermentation
- 4.6. Pressurized cultivation
- 4.7. Perfusion techniques
- 5. Induction conditions
 - 5.1. Quality of inducer
 - 5.2. Quantity of inducer
 - 5.3. Induction time
 - 5.4. Medium condition at induction phase
- 6. Process analysios and contro
- 7. Concluding remarks and future prospects

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),

1	1CDC
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

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

 UCDC

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), Kluvveromyces 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) Fed batch	Vuolanto <i>et al.</i> (2001)
Bacillus subtilis	35.6 g/l	Phytase	28.7 U/ml	(Controlled glucose concentration)	Kerovuo <i>et al.</i> (2000)
Bacillus subtilis	OD 60.1	Penicillin G acylase	1960 U/I	Fed batch (pH-stat)	Zhang <i>et al.</i> (2006)
Bacillus subtilis		Subtilisin	6 19 11/1	Fed batch (exponential feeding)	Oh et al. (2002)
Bacillus subtilis	184 g/l	β-galactosidase	157 g/l.day	Fed bach (pH-stat)	Park <i>et al</i> . (1992)
Saccharomyces cerevisiae					Formaira at al
cerevisiae	~ 45 g/l	Cutinase Human	1.6 g/l	Fed batch (pH-stat)	(2004a)
Saccharomyces cerevisiae	~ 45 g/l	granulocyte-colony stimulating factor	1.3 g/l	Fed batch (pH-stat) Fed batch	Lee <i>et al</i> . (1999)
Saccharomyces cerevisiae	120 g/l	Ergosterol Human	1500 mg/l	(Controlled glucose concentration)	Shang <i>et al.</i> (2006)
Saccharomyces cerevisiae Saccharomvces	53.6 g/l	granulocyte-colony stimulating factor	~150 mg/l	Fed batch (pH-stat)	Bae <i>et al</i> . (1999) Dominguez <i>et al.</i>
cerevisiae	50 g/l	β-galactosidase	6.28*10^5 U/lh	Continuous	(2005)
Pichia pastoris				Fed batch (mixed	
Pichia pastoris	125 g/l	Human cystatin_C Human	0.72 g/l	feeding)	Files <i>et al</i> . (2001)
Pichia pastoris	325 g/i (WCW)	IV	25 ma/l	Fed batch (DO-stat)	Li <i>et al.</i> (2003)
, Pichia pastoris	110 g/l	α-amylase	340 mg/l	Fed batch (DO-stat)	Lee et al. (2003) Chen et al
Pichia pastoris	OD 321	Phytase Bovine entrokinase light	4946 U/ml	Fed batch (DO-stat)	(2004)
Pichia pastoris	~ 45 g/l	chain	9000 U/ml	Fed batch (pH-stat)	(2004)
Ralstonia eutropha					
Ralstonia eutropha	113 g/l	Organophosphohy drolase	1353 U/mg	(changing the feed solution) Fed batch	Barnard <i>et al.</i> (2004)
Ralstonia eutropha	182 g/l	Organophosphohy drolase Poly(3-	1.2 g/l	(changing the feed solution)	Srinivasan <i>et al.</i> (2003)
Ralstonia eutropha	281 g/l	hydroxybutyrate) Poly(3-	232 g/l	Fed batch (DO-stat)	Ryu <i>et al</i> . (1997) Tsuge <i>et al</i>
Ralstonia eutropha	75 g/l	hydroxybutyrate) Poly(3-	54.8 g/l	Fed batch (pH-stat)	(2001)
Ralstonia eutropha	300 g/l	hydroxybutyrate)	97% recovery	Fed batch (DO-stat)	Kim <i>et al</i> . (2003)
Hansenula polymorpha Hansenula					
polymorpha Hansenula	~120 g/l	Levansucrase	12200 U/I	Fed batch (DO-stat)	Park <i>et al</i> . (2004)
polymorpha Hansenula	~35 g/l	Hirudin Human serum	144 mg/l	Fed batch (DO-stat) Fed batch	Kim <i>et al</i> . (1998)
polymorpha	83 g/l	albumin	550 mg/l	(intermittent feeding)	Heo <i>et al.</i> (2003)
Hansenula polymorpha	350 g/l (WCW)	Human α1 (I) procollagen	0.6 g/l	Fed batch (changing the feed solution)	de Bruin <i>et al.</i> (2000) Muller et al
nansenula polymorpha	OD 70	Interferon α-2a	350 mg/l	⊢eu baicn (pH-stat, DO-stat)	(2002)

65 www.SID.ir

Table 2. Continued.

Kluyveromyces marxianus					
Kluyveromyces marxianus	105 g/l	Biomass	2.9 g/l.h	Fed bacth (exponential feeding) Fed bacth	Bojorge <i>et al.</i> (1999)
Kluyveromyces marxianus	28.13 g/l	Oligonucleoides	2.42 g/l.h	(controlled lactose concentration)	Ferreira <i>et al.</i> (2004b) Balana 2 haor
Kluyveromyces marxianus	~60 g/l	Lactase	N/A	Fed batch (pH-stat) Fed batch	Belem & Lee (1999)
Kluyveromyces marxianus	~20 g/l	β-galactosidase	~3000 U/g	(stepwise nutrient feeding)	Lukondeh <i>et al.</i> (2005) Cortos et el
marxianus	12.2 g/l	β-galactosidase	2800 U/g	Batch	(2005)
Panax notoginseng				End botch (combined	Mong et el
Panax notoginseng	27.3 g/l	Ginseng saponin	290 mg/l	feeding)	(2005) Zhong <i>et al</i> .
Panax notoginseng	28.9 g/l	Ginseng saponin	0.92 g/l	Batch	(1999) Han & Zhong
Panax notoginseng Panax notoginseng	24 g/l 24 g/l	Ginseng saponin	1.75 g/l 1.7 g/l	Batch Batch	(2003)
Panax notoginseng	30 g/l 24.1 g/l	Ginseng saponin	2.3 g/l 1.4 g/l	Fed batch (SOUR based feeding) Batch	Han & ∠hong (2002)
	29.7 g/l	Ginseng saponin	2.1 g/l	Fed batch (SOUR based feeding)	Hu <i>et al</i> . (2001)
Taxus chinensis					
Taxus chinensis cells	22 g/l	Paclitaxel	67 mg/l	Fed batch (intermittent feeding) Fod batch (combined	Choi <i>et al.</i> (2000)
cells Taxus chinensis	22.7 g/l	Taxane	278 mg/l	feeding)	(2002)
cells Taxus chinensis	16.58 g/l	Taxane	5.4 mg/DCW	Batch	Pan <i>et al.</i> (2000) Zhong <i>et al</i>
cells Taxus chinensis	15.9 g/l	Taxoid	~70 mg/l	Batch	(2002) Wang <i>et al.</i>
cells	~17 g/l	Taxol	67.1 mg/l	Batch	(2001)
Spodoptera frugiperda					
Spodoptera fruciperda	5.2*10^7 cells/ml	ß-galactosidase	500 U/ml	Fed batch (pulse & semi continuous feedings)	Elias <i>et al.</i> (2000)
Spodoptera frugiperda	7*10^6 cells/ml	L1	200 ma/l	Batch	Gouveia <i>et al.</i> (2007)
Spodoptera frugiperda	7-9*10^6 cells/ml	Bovine rhodopsin	4 mg/l	Batch	Klaassen <i>et al.</i> (1999)
Spodoptera frugiperda	3*10^7 cells/ml	Human chitinase	N/A	Perfusion culture	Zhang <i>et al.</i> (1998)
Spodoptera frugiperda	8*10^ cells/ml	β-galactosidase	5-9.8*10^5 U/ml	Batch	Radford <i>et al.</i> (1997)
Tetrahymena thermophila					
Tetrahymena thermophila	54 g/L	N/A	N/A	Continuous	Kiy and Tiedtke (1992)
Tetrahymena thermophila Tetrahymena	cells/ml (48 g/l)	Some lysosomal enzymes	Different	Continuous with cell recycling	Kiy <i>et al.</i> (1996) Weide <i>et al</i>
thermophila Tetrahymena	48 g/L 1 9*10^6	Human DNase I	100 µg/L	Batch Continuous with cell	(2006) de Coninck <i>et al</i>
thermophila Tetrahymena	cells/ml 3*10^6	Protease	~900 mU/ml	recycling	(2000) Hellenbroich <i>et</i>
thermophila	cells/ml	Protease	~400 nkat/l	Batch	al. (1999)

Table 2. Continued.

Chinese hamster ovary cells					
Chinese hamster ovary cells	6.2*10^6 cells/ml	Interferon a	~20 mg/l	Fed batch (exponential feeding) Fed batch	Lim <i>et al.</i> (2006)
Chinese hamster ovary cells Chinese hamster	5*10^6 cells/ml 0 1*10^6	Human Antithrombin	1g/l	(controlled glucose concentration)	Kuwae <i>et al.</i> (2005) Meuwly <i>et al</i>
ovary cells	cells/ml	N/A	N/A	Perfusion	(2006)
Chinese hamster ovary cells Chinese hamster	3.71×10^ 6 cells/ml 2*10^9	Erythropoietin Angiostatin-human	121.2 µg/mL	Biphasic culture	Yoon <i>et al.</i> (2006) Wang <i>et al.</i>
ovary cells	cells/ml	lgG	130 mg/l	Semi-batch	(2005)
Escherichia coli					
		Human interferon		Fed batch (modified variable	Babaeinour <i>et al</i>
Escherichia coli	115 g/l	Y	42.5 g/l	specific growth rate) Fed batch	(2007)
Escherichia coli	101 g/l	Poly(3- hydroxybutyrate) Human interferon	4.4 g/l	(exponential feeding and pH-stat) Fed batch	Kim <i>et al</i> . (2004) Babu <i>et al</i>
Escherichia coli	~90 g/l	α	4 g/l	(exponential feeding) Fed batch	(2000)
Escherichia coli	162 (WCW)	Penicillin G Acylase	37 IU/WCW	(controlled sorbitol concentration) Fed batch	Liu <i>et al.</i> (2000)
Escherichia coli	80 g/l	Gluthathione	880 mg/l	(exponential feeding)	Li <i>et al</i> . (1998)
Other microorganisms					
Methylobacterium		Green fluorescent		Fed batch(OTR	Belanger <i>et al</i> .
extorquens	56 g/l	protein	4 g/L	based feeding)	(2004)
extorquens Methylobacterium	g/l	Poly(3- hydroxybutyrate) Poly-8-hydroxy	0.2 g/DCW	based feeding)	Bourque <i>et al.</i> (1995) Suzuki <i>et al.</i>
extorquens	233 g/l	butyric acid Eicosapentaenoic	32.9 g/l.day	Fed batch (DO-stat) Perfusion-cell	(1986) Wen and Chen
Nitzschia laevis	~25 g/l	acid Eicosapentaenoic	321 mg/l.day	bleeding culture Fed batch (glucose	(2001)
Nitzschia laevis	22.1 g/l	acid Eicosapentaenoic	695 mg/l	controlled)	Wen <i>et al</i> . (2002) Wen and Chen
Nitzschia laevis	40 g/l	acid	1112mg/l	Perfusion culture Fed-batch	(2002)
Bacillus clausii Trigonopsis	~60 g/l	Savinase	15 mg /DCW	(linear and exponential feeding) Two stage	Christiansen <i>et</i> al. (2003)
variabilis Crypthecodinium	34 g/l	Erythritol Docosahexaenoic	46 g/l	fermentation	Kim <i>et al</i> . (1997) De Swaaf <i>et al</i>
cohnii Galdieria	109 g/L 80-120	acid	19 g/L	Fed batch (DO-stat)	(2003) Schmidt et al
sulphuraria	g/L	Phycocyanin Di hama protoin	250-400 mg/l	Fed batch (DO-stat)	(2005)
putida	34 g/l	cytochrome c(4)	29 mg/l	(exponential feeding)	(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. col.
--

Method	Process description	Reference
Using different expression systems	<i>E. coli</i> B (which produces less acetate) were used instead of <i>E. coli</i> K	Noronha <i>et al</i> . (2000); Phue <i>et al</i> . (2005)
Mutants with defects in acetate biosynthetic pathway	Mutants of <i>E.coli</i> w3100 were generated which lacked acetate associated enzymatic activity and produced less acetate	Contiero <i>et al</i> . (2000)
Enhancing acetate utilization	Mutants were generated which could consume acetate as carbon source even in the presence of glucose	Oh <i>et al.</i> (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 <i>et al.</i> (1998) Aristidou <i>et al.</i> (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 pro- duction 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, nonnitrogen 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 limit-ed supply of carbon source. The main metabolic by-products are acetate for *E. coli*, propionate for *B. sub-tilis*, 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 KH₂PO₄ 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 (6deoxyerythronolide 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 trialand-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 25°C 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 fedbatch 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 fedbatch 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

Shojaosadati et al.

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) $(3.26 \times 10^{-3} \text{ to } 5.11 \times 10^{-2} \text{ mmol/g of cell})$ on production of mini-proinsulin and reported $5.11 \times 10^{-2} \text{ mmol/g of cells}$ as optimum concentration. Vidal *et al.* (2005) investigated the

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

Promoter	Example	Inducers	Advantages	Disadvantages
<i>T7</i> or <i>lac</i> -based promoters	<i>tac, trc, lac, lacUV5-T7</i> hybrid	Isopropyl-β-D-thio- galactopyranoside (IPTG)	Products are effec- tively 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 car- bon source	Difficult to use in large scale
Positively regulat- ed systems	arabinose-inducible <i>PBAD</i> promoter, Rhamnose- inducible <i>rha</i> BAD promoter			Product quality decreases as cell densi- ty increases
Starvation-induced promoters	Trp, phoA	Exhaustion of a specific substrate		Substrate exhaustion interferes with produc- tion, time of induction is not known
Heat-inducible promoters	λPL	Temperature shift		Temperature shift adversely affects pro- duction, 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 μ mol IPTG/1.

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-y 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 30°C 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 measurement, а 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 chro-

matography 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 packedbed 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.

References

- Akesson M, Hagander P, Axelsson JP (2001). Avoiding acetate accumulation in *E. coli* cultures using feedback control of glucose feeding. *Biotechnol Bioeng*. 73: 223-230.
- Aristidou AA, Sari, KY, Bennett GN (1995). Metabolic engineering of *E. coli* to enhance recombinant protein production through acetate reduction. *Biotechnol Prog.* 11: 470-475.
- Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, et al. (2003). Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol. 21: 150-156.
- Bae CS, Yang DS, Lee J, Park YH (1999). Improved process for production of recombinant yeast-derived monomeric human G-CSF. *Appl Microbiol Biotechnol*. 52: 338-344.
- Babaeipour V, Shojaosadati SA, Khalilkzadeh R, Maghsoudi N Tabandeh F (2008). A proposed feeding strategy for the overproduction of recombinant proteins in *E. coli. Appl Microbiol Biotechnol.* 49: 141-147.
- Babaeipour V, Shojaosadati SA, Robatjazi M, Khalilkzadeh R, Maghsoudi N (2007). Over-production of human interferon-γ by HCDC of recombinant *E. coli. Process Biochem.* 42: 112-117.
- Babu KR, Swaminathan S, Marten S, Khanna U, Rinas U (2000). Production of interferon- α in high cell density cultures of recombinant *E. coli* and its single step purification from refolded inclusion body proteins. *Appl Microbiol Biotechnol.* 53: 655-660.
- Bahrami A, Shojaosadati SA, Khalilzadeh R, Vasheghani Farahani E (2008). Two stage glycerol feeding for enhancement of recombinant hG-CSF production in a fed batch culture of *Pichia pastoris*. *Biotechnol Lett.* 30: 1081-1085.
- Baker KN, Rendall M, Patel A, Boyd P, Hoare M (2002). Rapid monitoring of recombinant protein products: a comparison of current technologies. *Trends Biotechnol*. 20: 149-156.
- Barnard GC, Henderson GE, Srinivasan S, Gerngross TU (2004). High level recombinant protein expression in *Ralstonia eutropha* using T7 RNA polymerase based amplification. *Protein Expr Purif.* 38: 264-271.
- Barreto MTO, Melo EP, Moreira JL, Carrondo MJT (1991). High cell density reactor for the production of *Lactobacillus plantarum*. *J Ind Microbiol Biot*. 7: 63-69.
- Bélanger L, Figueira MM, Bourque D, Morel L, Béland M, Laramée L, Groleau D, Míguez CB (2004). Production of heterologous protein by *Methylobacterium extorquens* in high cell density fermentation. *FEMS Microbiol Lett.* 231: 197-204.
- Belem MAF, Lee BH (1999). Fed-batch fermentation to produce oligonucleotides from *Kluyveromyces marxianus* grown on whey. *Process Biochem.* 34: 501-509.
- Belo I, Mota M (1998). Batch and fed-batch cultures of *E. coli* TB1 at different oxygen transfer rates. *Bioproc Eng.* 18: 451-455.
- Bermejo LL, Welker NE, Papoutsakis ET (1998). Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. *Appl Environ Microbiol.* 64: 1079-85.

- Bojorge N, Valdman, B, Acevedo F, Gentina JC (1999). A semistructured model for the growth and β-Galactosidase production by fed-batch fermentation of *Kluyveromyces marxianus*. *Bioproc Eng.* 21: 313-318.
- Bourque D, Pomerleau Y, Groleau D (1995). High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. *Appl Microbiol Biotechnol.* 44: 367-376.
- Brady CP, Shimp RL, Miles AP, Whitmore M, Stowers AW (2001). High-level production and purification of P30P2MSP119, an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expr Purif.* 23: 468-475.
- Castan A, Nasman A, Enfors SO (2002). Oxygen enriched air supply in *E. coli* processes: production of biomass and recombinant human growth hormone. *Enzyme Microb Tech.* 37: 847-854.
- Castrillo JI, Kaliterna J, Weusthuis RA, Dijken PJ, Pronk JT (1996). High-cell-density cultivation of yeasts on disaccharides in Oxygen-limited batch cultures. *Biotechnol Bioeng*. 49: 621-628.
- Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM (2002). Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris. *Curr Opin Biotechnol.* 13: 329-332.
- Chang CC, Ryu DD, Park CS, Kim JY, Ogrydziak DM (1998). Recombinant bioprocess optimization for heterologous protein production using two-stage, cyclic fed-batch culture. *Appl Microbiol Biotechnol.* 49: 531-537.
- Chattaway T, Demain AL, Stephanopoulos G (1992). Use of various measurements for biomass estimation. *Biotechnol Prog.* 8: 81-84.
- Chen CC, Wu PH, Huang CT, Cheng KJ (2004). A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. *Enzyme Microb Tech.* 35: 315-320.
- Choi HK, Kim SI, Son JS, Hong SS, Lee HS, Chung IS, Lee HJ (2000). Intermittent maltose feeding enhances paclitaxel production in suspension culture of *Taxus chinensis* cells. *Biotechnol Lett.* 22: 1793-1796.
- Choi J, Lee SY (1999)a. Efficient and economical recovery of Poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnol Bioeng.* 62: 546-553.
- Choi JI, Lee SY (1999)b. High-level production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant *Escherichia coli*. Appl Environ Microbiol. 65: 4363-4368.
- Choi JH, Keum KC, Lee SY (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Chem Eng Sci.* 61: 876-885.
- Choi SJ, Park DH, Jung KH (2001). Development and optimization of two-stage cyclic fed-batch culture for hG-CSF production using L-arabinose promoter in *E. coli. Bioproc Biosyst Eng.* 24: 51-58.
- Christiansen T, Michaelsen S, Wumpelmann M, Nielsen J (2003). Production of savinase and population viability of *Bacillus clausii* during high-cell-density fed-batch cultivations. *Biotechnol Bioeng.* 83: 344-352.

- Chung YC, Chien IL, Chang DM (2006). Multiple-model control strategy for a fed-batch high cell-density culture processing. *J Process Contr.* 16: 9-26.
- Clementschitsch F, Kern J, Ptschacher F, Bayer K (2005). Sensor combination and chemometric modeling for improved process monitoring in recombinant *E. coli* fed-batch cultivations. *J Biotechnol*. 120: 183-196.
- Contiero J, Beatty C, Kumari S, DeSanti CL, Strohl WR, Wolfe A (2000). Effects of mutations in acetate metabolism on high-cell-density growth of *E. coli. J Ind Microbiol Biotechnol.* 24: 421-30.
- Cortes G, Trujillo-Roldan MA, Ramirez OT, Galindo E (2005). Production of β -galactosidase by *Kluyveromyces marxianus* under oscillating dissolved oxygen tension. *Process Biochem*. 40: 773-778.
- Crowley J, Alison AS, Wood N, Linda M, McNeil H, McNeil B (2005). Monitoring a high cell density recombinant *Pichia pastoris* fed-batch bioprocess using transmission and reflectance near infrared spectroscopy. *Enzyme Microb Tech.* 36: 621-628.
- Cserjan-Puschmann M, Grabherr R, Striedner G, Clementschitsch F, Bayer K (2002). Optimizing Recombinant Microbial Fermentation Processes: an integrated approach. *Biopharm*. 26-34.
- Curless C, Fu K, Swank R, Manjares A, Fieschko J, Tsai L (1991). Design and evaluation of a two stage, cyclic recombinant fermentation process. *Biotechnol Bioeng*. 38: 1082-1092.
- Daly R, Hearn MT (2005). Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineering and production. *J Mol Recogn.* 18: 119-138.
- De Bruin EC, De Wolf FA, Laane NCM (2000). Expression and secretion of human a1(I) procollagen fragment by *Hansenula polymorpha* as compared to *Pichia pastoris*. *Enzyme Microb Tech*. 26: 640-644.
- De Coninck J, Bouquelet S, Dumortier V, Duyme F, Verdier-Denantes I (2000). Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila* BIII. *J Ind Microbiol Biotechnol*. 24: 285-290.
- De Swaaf ME, Sijtsma L, Pronk JT (2003). High-Cell-Density Fed-Batch Cultivation of the Docosahexaenoic Acid Producing Marine Alga *Crypthecodinium cohnii*. *Biotechnol Bioeng*. 81: 666-672.
- Domingues L, Lima N, Teixeira JA (2005). *Aspergillus niger* βgalactosidase production by yeast in a continuous high cell density reactor. *Process Biochem*. 40: 1151-1154.
- Domingues L, Lima N, Teixeira JA (2000). Contamination of a High Cell-Density Continuous Bioreactor. *Biotechnol Bioeng*. 68: 584-587.
- Dong HD, Zhong JJ (2002). Enhanced taxane productivity in bioreactor cultivation of *Taxus chinensis* cells by combining elicitation, sucrose feeding and ethylene incorporation. *Enzyme Microb Tech.* 31: 116-121.
- Donovan RS, Robinson CW, Glick BR (1996). Review: optimizing inducer and culture conditions for expression of foreign proteins under the control of the Lac promoter. *J Ind Microbiol*. 16: 145-154.
- Elias CB, Zeiser A, Bedard C, Kamen AA (2000). Enhanced

growth of Sf-9 cells to a maximum density of 5.2×10^7 cells per mL and production of β -Galactosidase at high cell density by fed batch culture. *Biotechnol Bioeng.* 68: 381-8.

- Enfors SO, Jahic M, Rozkov A, Xu B, Hecker M, Jurgen B, Kruger E, Schweder T, Hamer G, O'Beirne D, Noisommit-Rizzi N, Reuss M, *et al.* (2001). Physiological Responses to Mixing in Large Scale bioreactors, *J Biotechnology*. 85: 175-185.
- Farmer WR, Liao JC (1997). Reduction of aerobic acetate production by *Escherichia coli*. *Appl Environ Microbiol*. 63: 3205-10.
- Files D, Ogawa M, Scaman CH, Baldwin SA (2001). A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C. *Enzyme Microb Tech.* 29: 335-340.
- Forrer K, Hammer S, Helk B (2004). Chip-based gel electrophoresis method for the quantification of half-antibody species in IgG4 and their by- and degradation products. *Anal Biochem*. 334: 81-88.
- Fuchs C, Köster D, Wiebusch S, Mahr K, Eisbrenner G, Märkl H (2002). Scale-up of dialysis fermentation for high cell density cultivation of *Escherichia coli*. J Biotechnology. 93: 243-251.
- Galvanauskas V, Simtis R, Lubbert A (1997). Model-based design of biochemical processes: simulation studies and experimental tests. *Biotechnol lett.* 10: 1043-1047.
- Gombert AK, Kilikian BV (1998). Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. J *Biotechnology*. 60: 47-54.
- Gorgens JF, van Zyl WH, Knoetze JH, Hahn-Hägerdal B (2005). Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. *Appl Microbiol Biotechnol.* 67: 684-691.
- Gouveia RM, Morais VA, Peixoto C, Sousa M, Regalla M, Alves PM, Costa J (2007). Production and purification of functional truncated soluble forms of human recombinant L1 cell adhesion glycoprotein from *Spodoptera frugiperda* Sf9 cells. *Protein Expr Purif.* 52: 182-193.
- Grabherr R, Nilsson E, Striedner G, Bayer K (2002). Stabilizing plasmid copy number to improve recombinant protein production. *Biotechnol Bioeng*, 77: 142-147.
- Graumann K, Premstaller A (2006). Manufacturing of recombinant therapeutic proteins in microbial systems. *J Biotechnol*. 1: 164-186.
- Guzman LM, Belin B, Carson MJ, Beckwith J (1995). Tight regulation, modulation and high level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol*. 177: 4121-4130.
- Haddadin FT, Tarcum SW (2005). Transcriptome Profiles For High-Cell-Density Recombinant and Wild-Type *E. coli. Biotechnol Bioeng.* 90: 127-53.
- Han J, Zhong JJ (2002). High density cell culture of *Panax notoginseng* for production of ginseng saponin and polysaccharide in an airlift bioreactor. *Biotechnol Lett.* 24: 1927-1930.
- Han J, Zhong JJ (2003). Effects of oxygen partial pressure on cell growth and ginsenoside and polysaccharide production in high density cell cultures of *Panax notoginseng*. *Enzyme Microb Tech.* 32: 498-503.

- Heim S, Ferrer M, Heuer H, Regenhardt D, Nimtz M, Timmis K.N. (2003). Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase. *Environ Microbiol.* 5: 1257-1269.
- Hellenbroich D, Valley U, Ryll T, Wagner R, Tekkanat N, Kessler W, Ross A, Deckwer WD (1999). Cultivation of Tetrahymena thermophila in a 1.5-m3 airlift bioreactor. *Appl Microbiol Biotechnol.* 51 :447-55.
- Helmann JD, Wu MF, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, Paddon C (2003). The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. *J Bacteriol*. 185: 243-253.
- Hensing M, Vrouwenvelder H, Hellinga C, Baartmans R, van Dijken H (1994). Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces marxianus*. *Appl Microbiol Biot*. 42: 516-521.
- Heo JH, Hong WK, Cho EY, Kim MW, Kim JY, Kim CH, Rhee SK, Kang HA (2003). Properties of the *Hansenula polymorpha*-derived constitutive *GAP* promoter, assessed using an HSA reporter gene. *FEMS Yeast Res.* 4: 175-184.
- Hermann T (2004). Using functional genomics to improve productivity in the manufacture of industrial biochemicals. *Curr Opin Biotech.* 15: 1-5.
- Hoeks FW, Boon LA, Studer F, Wolff MO, Van der Schot F, Vrabel
 P, Van der Lans RG, Bujalski W, Manelius A, Blomsten G,
 Hjorth S, Prada G, Luyben KC, Nienow AW (2003). Scale-up of stirring as foam disruption (SAFD) to industrial scale. *J Ind Microbiol Biot*. 30: 118-128.
- Hoffmann F, Schmidt M, Rinas U (2000). Simple Technique for Simultaneous On-Line Estimation of Biomass and Acetate from Base Consumption and Conductivity Measurements in High-Cell Density Cultures of *E. coli. Biotechnol Bioeng.* 70: 358-361.
- Hu WW, Yao H, Zhong JJ (2001). Improvement of *Panax notogin*seng Cell Culture for Production of Ginseng Saponin and Polysaccharide by High Density Cultivation in Pneumatically Agitated Bioreactors. *Biotechnol Prog.* 17: 838-846.
- Jahic M, Wallberg F, Bollok M, Garcia P, Enfors SO (2003). Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories*. 2: 6-17.
- Jana J, Deb JK (2005). Strategies for efficient production of heterologous proteins in *E. coli. Appl Microbiol Biotechnol.* 67: 289-298.
- Jensen EB, Carlsen S (1990). Production of recombinant human growth hormone in *E. coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. *Biotechnol Bioeng.* 36: 1-11.
- Jeong KJ, Choi JH, Yoo WM, Keum KC, Yoo NC, Lee SY, Sungf MH (2004). Constitutive production of human leptin by fedbatch culture of recombinant *rpoS E. coli. Protein Expres Purif.* 36: 150-156.
- Jeong KJ, Lee SY (2003). Enhanced production of recombinant proteins in *E. coli* by filamentation suppression. *Appl Environ Microb*. 69: 295-1298.
- Jin S, Shimizu YKK (1997). Metabolic flux distributions in recom-

binant *Saccharomyces cerevisiae* during foreign protein production. *J Biotechnol.* 54: 161-174.

- Kapat A, Jung JK, Park YH, Hong SY, Choi HK (1998). Effects of agitation and aeration on the production of extracellular glucose oxidase from a recombinant *Saccharomyces cerevisiae*. *Bioproc Eng.* 18: 347-351.
- Kellerhals MB, Kessler B, Witholt B (1999). Closed-Loop Control of Bacterial High-Cell-Density Fed-Batch Cultures: Production of mcl-PHAs by Pseudomonas putida KT2442 Under Single-Substrate and Cofeeding Conditions. *Biothechnol Bioeng*. 65: 306-315.
- Kerovuo J, Von Weymarn N, Povelainen M, Auer S, Miasnikov A (2000). A new efficient expression system for *Bacillus* and its application to production of recombinant phytase *Biotechnol Lett.* 22: 1311-1317.
- Khalilzadeh R, Shojaosadati SA, Bahrami A, Maghsoudi N (2003). Overexpression of recombinant human interferon-gamma in high cell density fermentation of *E. coli. Biotechnol Lett.* 25: 1989-1992.
- Khalilzadeh R, Shojaosadati SA, Maghsoudi N, Mohammadian-Mosaabadi J, Mohammadi MR, Bahrami A, Maleksabet N, Nassiri-Khalilli MA, Ebrahimi M, Naderimanesh H (2004).
 Process development for Production of recombinant human interferon-γ-expressed in *E. coli. J Ind Microbiol Biotechnol.* 31: 63-69.
- Kim SY, Lee KH, Kim JH, Oh DK (1997). Erythritol production by controlling osmotic pressure in *Trigonopsis variabilis*. *Biotechnol Lett.* 19: 727-729.
- Kim CH, Seo HW, Kim HY, Sohn JH, Choi ES, Rhee SK (1998). Production of recombinant hirudin in *Hansenula polymorpha*: variation of gene expression level depends on methanol oxidase and fermentation strategies. *J Ind Microbiol Biotechnol*. 21: 1-5.
- Kim M, Cho KS, Ryu HW, Lee EG, Chang YK (2003). Recovery of poly(3-hydroxybutyrate) from high cell density culture of *Ralstonia eutropha* by direct addition of sodium dodecyl sulfate. *Biotechnol Lett.* 25: 55-59.
- Kim BS, Lee SC, Lee SY, Chang YK, Chang HN (2004). High cell density fed-batch cultivation of *Escherichia coli* using exponential feeding combined with pH-stat. *Bioprocess Biosyst Eng.* 26: 147-150.
- Kim BS, Lee SC, Lee SY (1994). Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnol Bioeng.* 43: 892-898.
- Kim JYH, Cha HJ (2003). Down-Regulation of Acetate Pathway Through Antisense Strategy in *E. coli*: Improved Foreign Protein Production. *Biotechnol Bioeng.* 83: 841-853.
- Kiy T, Tiedtke A (1992). Continuous high-cell-density fermentation of the ciliated protozoon *Tetrahymena* in a perfused bioreactor. *Appl Microbiol Biotechnol*. 38:141-146.
- Kiy T, Scheidgen-Kleyboldt G, Tiedtke A (1996). Production of lysosomal enzymes by continuous high-cell-density fermentation of the ciliated protozoon *Tetrahymena thermophila* in a perfused bioreactor. *Enzyme Microb Technol.* 18: 268-274.
- Klaassen CHW, Bovee-geurts PHM, De Caluwe GLJ, De Grip WJ (1999). Large-scale production and purification of functional recombinant bovine rhodopsin with the use of the baculovirus

expression system. J Biochem. 342: 293-300.

- Kleman G, Strohl W (1994). Acetate metabolism by *Escherichia coli* in high cell density fermentation. *Appl Environ Microbiol*. 60: 3952-3958.
- Konstantinov K, Kishimoto M, Seki T, Yoshida T (1990). A balanced DO-stat and its application to the control of acetic acid excretion by recombinant *E. coli. Biotechnol Bioeng.* 70: 253-260.
- Kuwae S, Ohda T, Tamashima H, Miki H, Kobayashi K (2005). Development of a fed-batch culture process for enhanced production of recombinant human antithrombin by Chinese hamster ovary cells. *J Biosci Bioeng*. 100:502-10.
- Laffend L, Shuler ML (1994). Ribosomal protein limitations in *E. coli* under conditions of high translational activity. *Biotechnol Bioeng.* 43: 388-398.
- Lau J, Tran C, Licari P, Galazzo J (2004). Development of a high cell-density fed-batch bioprocess for the heterologous production of 6-deoxyerythronolide B in *E. coli. J Biotechnology*. 110: 95-103.
- Lee J, Lee SY, Park S, Middelberg AP (1999). Control of fed-batch fermentations. *Biotechnol Adv.* 17: 29-48.
- Lee J, Ramirez WF (1992). Mathematical modeling of induced foreign protein production by recombinant bacteria. *Biotechnol Bioeng*. 39: 635-646.
- Lee J, Choi SI, Jang JS, Jang K, Moon JW, Bae CS, Yang DS, Seong BL (1999). Novel secretion system of recombinant *Saccharomyces cerevisiae* using an N-terminus residue of Human IL-1, as secretion enhancer. *Biotechnol Prog.* 15: 884-890.
- Lee CY, Nakano A, Shiomi N, Lee EK, Katoh S (2003). Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. *Enzyme Microb Tech.* 33: 358-365.
- Lee SY (1994). Suppression of filamentation in recombinant *Escherichia coli* by amplified ftsZ activity. *Biotechnol Lett.* 16: 1247-1252.
- Lee SY, Chang HN (1993). High cell density cultivation of *E. coli* using sucrose as a carbon source. *Biotechnol Lett.* 15: 971-974.
- Lee SY, Wong HH, Choi J, Lee SH, Lee SC, Han CS (2000). High-Cell-Density Cultivation of *Pseudomonas putida* Under Phosphorus Limitation. *Biotechnol Bioeng*. 68: 466-70.
- Lee SY (1996). High cell density cultivation of *E. coli. Trends Biotechnol.* 14: 98-105.
- Lim SF, Chuan KH, Liu S, Loh SOH, Chung BYF, Ong CC, Song, Z (2006). RNAi suppression of Bax and Bak enhances viability in fed-batch cultures of CHO cells. *Metabol Eng.* 8: 509-522.
- Li X, Robbins JW, Taylor KB (1990). The production of recombinant β-galactosidase in *E. coli* in yeast extract enriched medium. *J Ind Microbiol*. 5: 690-697.
- Li Y, Chen J, Mao Y, Lun S, Koo Y (1998). Effect of additives and fed batch culture strategies on the production of glutathione by recombinant *Escherichia coli*. *Process Biochem*. 33: 709-714.
- Li A,Crimmins DL, Luo Q, Hartupee J, Landt Y, Ladenson JH, Wilson D, Anant S, Dieckgraefe BK (2003). Expression of a novel regenerating gene product, Reg IV, by high density fer-

mentation in *Pichia pastoris*: production, purification, and characterization. *Protein Expr Purif.* 31: 197-206.

- Lin H, Hoffmann F, Rozkov A, Enfors SO, Rinas U, Neubauer P (2004). Change of Extracellular cAMP Concentration Is a Sensitive Reporter for Bacterial Fitness in High-Cell-Density Cultures of *Escherichia coli*. *Biotechnol Bioeng*. 87: 602-613.
- Liu YC, Liao LC, Wu WT (2000). Cultivation of recombinant *Escherichia coli* to achieve high cell density with a high level of Penicillin G Acylase activity. *Proc Natl Sci Counc* 24: 156-160.
- Lukondeh T, Ashbolt NJ, Rogers PL (2005). Fed-batch fermentation for production of *Kluyveromyces marxianus*FII 510700 cultivated on a lactose-based medium. *J Ind Microbiol Biotechnol.* 32: 284-288.
- Madurawe RD, Madurawe RD, Chase TE, Tsao EI, Bentley WE (2000). A recombinant lipoprotein antigen against lyme disease expressed in *E. coli*: fermentor operating strategies for improved yield. *Biotechnol Prog.* 16: 571-576.
- Makrides SC (1996). Strategies for achieving high-level expression of genes in *E. coli. Microbiol Rev.* 60: 512-538.
- Mare Ld, Velut S, Ledung E, Cimander C, Norrman B, Karlsson EN, Holst O, Hagander P (2005). A cultivation technique for *E. coli* fed-batch cultivations operating close to the maximum oxygen transfer capacity of the reactor. *Biotechnol Lett.* 27: 983-990.
- Markl H, Zenneck C, Dubach A, Ogbonna JC (1993). Cultivation of *E. coli* to high cell densities in a dialysis reactor. *Appl Microbiol Biot*. 39: 48-52.
- Marose S, Lindemann C, Ulber R, Scheper T (1999). Optical sensor systems for bioprocess monitoring. *Trends Biotechnol*. 17: 30-34.
- Matsui T, Shinzato N, Yokota H, Takahashi J, Sato S (2006). High cell density cultivation of recombinant *E. coli* with a pressurized culture. *Process Biochem.* 41: 920-924.
- Mergulhao FJM, Summers DK, Monteiro GA (2005). Recombinant protein secretion in *E. coli. Biotechnol Adv.* 23: 177-202.
- Meuwly F, Papp F, Ruffieux PA, Bernard AR, Kadouri A, Stockar UV (2006). Use of glucose consumption rate (GCR) as a tool to monitor and control animal cell production processes in packed-bed bioreactors. *J Biotechnol*. 122: 122-129.
- Moon H, Kim SW, Lee J, Rhee SK, Choi ES, Kang HA, Kim IH, Hong SI (2004). Independent Exponential Feeding of Glycerol and Methanol for Fed-Batch Culture of Recombinant *Hansenula polymorpha* DL-1. *Glycobiology*. 14: 243-51.
- Muller FII, Tieke A, Waschk D, Muhle C, Muller FI, Seigelchifer M, Pesce A, Jenzelewski , Gellissen G (2002). Production of IFNα -2a in *Hansenula polymorpha*. Process Biochem. 38: 15-25.
- Nacib N, Branlant H, Boudrant C (1991). Metabolic roles of peptone and yeast extract for the culture of a recombinant strain of *Escherichia coli*. *J Ind Microbiol*. 8: 165-170.
- Nakano MM, Dailly YP, Zuber P, Clark DP (1997). Characterization on anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J Bacteriol. 179: 6749-6755.

Neubauer P, Hofmann K, Holst O (1992). Maximizing the expres-

sion of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose as inducer. *Appl Microbiol Biotechnol.* 36: 739-744.

- Noronha SB, Yeh HJ, Spande TF, Shiloach J (2000). Investigation of the TCA cycle and the glyoxylate shunt in *Escherichia coli* BL21 and JM109 using (13) C-NMR/MS. *Biotechnol Bioeng*. 68: 316-327.
- Oh JH, Kim BG, Park TH (2002). Importance of specific growth rate for subtilisin expression in fed-batch cultivation of *Bacillus subtilis spoIIG* mutant . *Enzyme Microb Tech.* 30: 747-751.
- Oh, M.K., Rohlin, L., Kao, K.C., and Liao, J.C. 2002. Global expression profiling of acetate-grown *E. coli. J Biol Chem.* 277: 13175-13183.
- Paalme T, Elken R, Vilu R, Korhola M (1997). Growth efficiency of *Saccharomyces cerevisiae* on glucose/ethanol media with a smooth change in the dilution rate (A-stat). *Enzyme Microb Tech.* 20: 217-230.
- Paalme T, Kahru A, Elken R, Vanatalu K, Tiisma K, Vilu R (1995). The computer-controlled continuous culture of *Escherichia coli* with smooth change of dilution rate. *J Microbiol Meth.* 24: 145-153.
- Paalme T, Vilu R (1992). A new method of continuous cultivation with computer-controlled change of dilution rate. In: *Modeling and control of biotechnical processes*. Karin MN, Stephanopolous G Oxford: Permagon Press. PP. 299-301
- Pan ZW, Wang HO, Zhong JJ (2000). Scale-up study on suspension cultures of *Taxus chinensis* cells for production of taxane diterpene. *Enyme Microb Tech.* 27: 714-723.
- Park YS, Kai K, Iijima S, Kobayashi T (1992). Enhanced ß-galactosidas production by high cell density culture of recombinant *Bacillus subtilis* with glucose concentration control. *Biotechnol Bioeng.* 40: 686-696.
- Park BS, Vladimir A, Kim CH, Rhee SK, Kang HA (2004). Secretory production of *Zymomonas mobilis* levansucrase by the methylotrophic yeast *Hansenula polymorpha*. *Enzyme Microb Tech.* 34: 132-138.
- Peng L, Zhong X, Ou J, Zheng S, Liao J, Wang L, Xu A (2004). High-level secretory production of recombinant bovine enterokinase light chain by *Pichia pastoris. J Biotechnol.* 108: 185-192.
- Peuker T, Riedel M, Kaiser C, Ellert A, Lenz K, Elsholz O, Luttmann R (2004). At-line determination of glucose, ammonia, and acetate in high cell density cultivations of *E. coli. Eng Life Sci.* 4: 138-143.
- Phue J, Noronha SB, Hattacharyya R, Wolfe AJ, Shiloach J (2005). Glucose Metabolism at High Density Growth of *E. coli* B and *E. coli* K: Differences in Metabolic Pathways Are Responsible for Efficient Glucose Utilization in *E. coli* B as Determined by Microarrays and Northern Blot Analyses; *Biotechnol Bioeng.* 90: 805-820.
- Prytz I, Sanden AM, Nystrom T, Farewell A, Wahlstrom A, Forberg C, Pragai Z, Barer M, Harwood C, Larsson LG (2003). Fed-Batch Production of Recombinant beta-Galactosidase Using the Universal Stress Promoters *uspA* and *uspB* in High Cell Density Cultivations. *Biotechnol Bioeng*. 83: 595-603.
- Radford, K.M., Cavegn, C., Bertrand, M., Bernard, A.R., Reid, S.,

and Greenfield, P.F. 1997. The indirect effects of multiplicity of infection on baculovirus expressed proteins in insect cells: secreted and non-secreted products. *Cytotechnology*. 24: 73-81.

- Raman B, Nandakumar MP, Muthuvijayan V, Marten MR (2005). Proteome analysis to assess physiological changes in *Escherichia coli* grown under glucose-limited fed-batch conditions. *Biotechnol Bioeng*, 92: 384-392.
- Ramirez DM, Bentley WE (1993). Characterization of stress and protein turnover from protein overexpression in fed-batch *E.coli* cultures. *J Biotechnology*. 71: 39-58.
- Resina D, Cos O, Ferrer, P, Valero F (2005). Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen sourceregulated FLD1 promoter. *Biotechnol Bioeng*. 91: 760-767.
- Riesenberg D, Guthke R (1999). High cell density cultivation of microorganism. *Appl Microbiol Biotechnol.* 51: 422-430.
- Rocha I, Ferreira EC (2002). On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli. Analytica Chimica Acta*. 462: 293-304.
- Rozkov A, Han L, Haggstrom L, Enfors SO (2001). Effects of addition of amino acids on growth of *E. coli* and production of recombinant protein, *Ph.D. Thesis*. Dept of Biootehnology, Royal Institute of Technology, Sweden.
- Ruckert C, Pühler A, Kalinowski J (2003). Genome-wide analysis of the L-methionine biosynthetic pathway in *Corynebacterium glutamicum* by targeted gene deletion and homologous complementation. *J Biotechnol*. 104: 213-228.
- Ryu HW, Hahn SK, Chang YK, Chang HN (1997). Production of Poly(3-hydroxybutyrate) by high cell density fed-batch culture of *Alcaligenes eutrophus* with phosphate limitation. *Biotechnol Bioeng.* 55: 28-32.
- Salusjarvi L, Poutanen M, Pitkanen JP, Koivistoinen H, Aristidou A, Kalkkinen N, Ruohonen L, Penttila M (2003). Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*. Yeast 20: 295-314.
- Scheidgen-Kleyboldt G, Kuchta K, Kiy T (2003). Production of secreted hydrolytic enzymes by continuous high-cell-density cultivation of *Colpidium campylum*. *Eur J Protistol*. 39: 455-460.
- Schmidt RA, Wiebe MG, Eriksen NT (2005). Heterotrophic High Cell-Density Fed-Batch Cultures of the Phycocyanin-Producing Red Alga *Galdieria sulphuraria*. *Biotechnol Bioeng*. 90: 77-84.
- Shang F, Wen S, Wang X, Tan T (2006). High-cell-density fermentation for ergosterol production by *Saccharomyces cerevisiae*. *J Biosci Bioeng*. 101: 38-41.
- Shiloach JA, Fass R (2005). Growing *E.coli* to high cell density-A historical perspective on method development. *Biotechnol Adv*. 23: 345-357.
- Shimizu H, Miura K, Shioya S, Suga K (1993). An overview on the control system design of bioreactors. Adv Biochem Eng Biotechnol. 50: 65-84.
- Shin CS, Hong MS, Bae CS, Lee J (1997). Enhanced production of human Miniproinsulin in fed-batch cultures at high cell density of *E. coli* BL21 (DE3)(pET-3aT2m2). *Biotechnol Prog.* 3: 249-257.

- Shokri A, Larsson G (2004). Characterization of *E. coli* membrane structure and function during fedbatch cultivation. *Microbial Cell Factories*. 3: 9-21.
- Shuler ML, Kargi F (1992). *Bioprocess engineering, Basic concepts*. New Jersey: Prentice Hall.
- Sorensen HP, Mortensen KK (2005). Advanced genetic strategies for recombinant protein expression in *E. coli. J Biotechnology.* 115: 113-128.
- Srinivasan S, Barnard GC, Gerngross TU (2003). Production of Recombinant Proteins Using Multiple-Copy Gene Integration in High-Cell-Density Fermentations of *Ralstonia eutropha*. *Biotechnol Bioeng*. 84: 114-120.
- Strandberg, L., Enfors, S.O. 1991. Batch and fed batch cultivations for the temperature induced production of a recombinant protein in *Escherichia coli*. *Biotechnol lett.* 13: 609-614.
- Striedner G, Cserjan-Puschmann M, Potschacher F, Bayer K (2003). Tuning the transcription rate of recombinant protein in strong *Escherichia coli* expression systems through repressor titration, *Biotechnol Prog.* 19: 1427-1432.
- Su WS, Arias R (2003). Continuous plant cell perfusion culture : bioreactor characterization and secreted enzyme production. *J Biosci Bioeng*. 95: 13-20.
- Suzuki T, Yamane T, Shimizu S (1986). Mass production of polyb-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. *Appl Microbiol Biotechnol*. 24: 370-374.
- Svensson M, Han L, Silfversparre G, Haggstrom L, Enfors SO (2005). Control of endotoxin release in *Escherichia coli* fedbatch cultures. *Bioproc Biosyst.* 27: 91-97.
- Tabandeh F, Shojaosadati SA, Zomorodipour A, Khodabandeh M, Sanati MH, Yakhchali B (2004). Heat-induced production of human growth hormone by high cell density cultivation of recombinant *E. coli. Biotechnol lett.* 26: 245-250.
- Tashiro Y, Takeda K, Kobayashi G, Sonomoto K (2005). High production of acetone-butanol-ethanol with high cell density culture by cell-recycling and bleeding. *J Biotechnol*. 120: 197-206.
- Thuesen HM, Norgaard A, Hansen AM, Caspersen MB, Christensen HEM (2003). Expression of recombinant *Pseudomonas stutzeri* di-heme cytochrome c_4 by high-celldensity fed-batch cultivation of *Pseudomonas putida*. *Protein Expres Purif.* 27: 175-181.
- Ting TE, Thoma GJ, Beitle Jr RR, Davis RK, Perkins R, Karim K Liu HM (2008). A simple substrate feeding strategy using a pH control trigger in fed-batch fermentation. 149: 89-98.
- Tsuge T, Tanaka K, Ishizaki A (2001). Development of a novel method for feeding a mixture of L-lactic acid and acetic acid in fed-batch culture of *Ralstonia eutropha* for Poly-D-3-Hydroxybutyrate production. *J Biosci Bioeng*. 91: 545-550.
- Vallejo LF, Brokelmann M, Marten S, Trappe S, Cabrera-Crespo J, Hoffmann A, Gross G, Weich HA, Rinas U (2002). Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*. J Biotechnology. 94: 185-194.
- van Hoek P, de Hulster E, van Dijken JP, Pronk JT (2000). Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. *Biotechnol Bioeng*. 68: 517-523.
- Vasilyeva E, Woodard J, Taylor FR, Kretschmer M, Fajardo H,

Lyubarskaya Y, Kobayashi K, Dingley A, Mhatre R (2004). Development of a chip-based capillary gel electrophoresis method for quantification of a half-antibody in immunoglobulin G4 samples. *Electrophoresis* 25: 3890-3896.

- Vidal L, Ferrer P, Alvaro G, Benaiges MD, Caminal G (2005). Influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by *E. coli* using the T5 promoter. *J Biotechnol*. 118: 75-87.
- Vido K, Le Bars D, Ves Mistou MY, Anglade P, Gruss A, Gaudu P (2004). Proteome Analyses of Heme-Dependent Respiration in *Lactococcus lactis*: Involvement of the Proteolytic System. *J Bacteriol*. 186: 1648-1657.
- Viitanen MI, Vasala A, Neubauer P, Alatossava T (2003). Cheese whey-induced high cell-density production of recombinant proteins in *E. coli. Microbial Cell Factories*. 2-12.
- Vuolanto A, von Weymarn N, Kerovuo J, Ojamo H, Leisola M (2001). Phytase production by high cell density culture of recombinant *Bacillus subtilis*. *Biotechnol Lett.* 23: 761-766.
- Wang F, Lee SY (1998). High cell density culture of metabolically engineered *E. coli* for the production of poly (3-hydroxybutyrate) in a defined medium. *Biotechnol Bioeng*. 58: 325-328.
- Wang C, Wu J, Mei X (2001). Enhancement of Taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. *Appl Microbiol Biotechnol*. 55: 404-410.
- Wang W, Zhang ZY, Zhong JJ (2005). Enhancement of ginsenoside biosynthesis in high-density cultivation of *Panax notoginseng* cells by various strategies of methyl jasmonate elicitation. *Appl Microbiol Biotechnol.* 67: 752-758.
- Weide T, Herrmann L, Bockau U, Niebur N, Aldag I, Laroy W, Contreras R, Tiedtke A, Hartmann MWW (2006). Secretion of functional human enzymes by *Tetrahymena thermophila*. *BMC Biotechnol.* 6: 19-28.
- Weikert C, Sauer U, Baily JE (1998). An *Escherichia coli* host strain useful for efficient overproduction of secreted recombinant protein. *Biotechnol Bioeng*. 59: 386-91.
- Wen ZY, Jiang Y, Chen F (20020. High cell density culture of the diatom *Nitzschia laevis* for eicosapentaenoic acid production: fed-batch development. *Process Biochem.* 37: 1447-1453.
- Wen ZW, Chen F (2001). A perfusion–cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis. Appl Microbiol Biotechnol.* 57: 316-322.
- Wen ZW, Chen F (2002). Perfusion culture of the diatom Nitzschia laevis for ultra-high yield of eicosapentaenoic acid. Process Biochem. 38: 523-529.
- Werner R (2004). Economic aspects of commercial manufacture of biopharmaceuticals, *J Biotechnolog*. 113: 171-182.
- Whiffin VS, Cooney MJ, Cord-Ruwisch R (2004). Online detection of feed demand in high cell density cultures of *E. coli* by measurement of changes in dissolved Oxygen transients in complex media. *Biotechnol Bioeng.* 85: 422-33.
- Whitney GK, Glick BR, Robinson CW (1989). Induction of T4 DNA ligase in a recombinant strain of *E. coli. Biotechnol Bioeng.* 22: 991-998.
- Wilms B, Hauck A, Reuss M, Syldatk C, Mattes R, Siemann M, Altenbuchner J (2001). High-cell-density fermentation for production of L-N-Carbamoylase using an expression system based on the *Escherichia coli* rhaBAD promoter. *Biotechnol*

Bioeng. 73: 95-103.

- Winzer K, Hardie KR, Williams P (2002). Bacterial cell to cell communication: sorry, can't talk now- gone to lunch. *Curr Opin Microbiol.* 5: 216-22.
- Yang J, Angelillo Y, Chaudhry M, Goldenberg C, Goldenberg DM (2000). Achievement of high cell density and high antibody productivity by a controlled-fed perfusion bioreactor process; *Biotechnol Bioeng*. 69: 74-82.
- Yee L, Blanch HW (1993). Recombinant trypsin production in high cell density fed-batch cultures in *E. coli. Biotechnol Bioeng*, 41: 781-790.
- Yoon SH, Han MJ, Lee SY, Jeong KJ, Yoo JS (2003). Combined transcriptome and proteome analysis of *Escherichia coli* during high cell density culture. *Biotechnol Bioeng*. 81: 753-767.
- Ho YS, Han MJ, Lee SY, Jeong KJ, Yoo JS (2003). Combined transcriptome and proteome analysis of *E. coli* during high cell density culture. *Biotechnol Bioeng*. 81: 753-67.
- Zhang J, Collins A, Chen M, Knyazev I, Gentz R (1998). Highdensity perfusion culture of insect cells with a BioSep ultrasonic filter. *Biotechnol Bioeng*. 59: 351-359.
- Zhang W, Li ZJ, Agblevor FA (2005). Microbubble fermentation of recombinant Pichia pastoris for human serum albumin production. *Process Biochem*. 40: 2073-2078.
- Zhang M, Shi M, Zhou Z, Yang S, Yuan Z, Ye Q (2006). Production of *Alcaligenes faecalis* penicillin G acylase in *Bacillus subtilis* WB600 (pMA5) fed with partially hydrolyzed starch. *Enzyme Microb Tech.* 39: 555-560.
- Zhong JJ, Chen F, Hu WW (1999). High density cultivation of *Panax notoginseng* cells in stirred bioreactors for the production of ginseng biomass and ginseng saponin. *Process Biochem.* 35: 491- 496.
- Zhong JJ, Pan ZW, Wang ZY, Wu J, Chen F, Takagi M, Yoshida T (2002). Effect of mixing time on Taxoid production using suspnsion cultures of *Taxus chinensis* in a centrifugal impeller bioreactor. *J Biosci Bioeng*. 94: 244-250.