



Review

Bioreactor engineering for recombinant protein production in plant cell suspension cultures

Ting-Kuo Huang, Karen A. McDonald*

Department of Chemical Engineering and Materials Science, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

ARTICLE INFO

Article history:

Received 31 July 2008

Received in revised form 16 February 2009

Accepted 17 February 2009

Keywords:

Recombinant proteins

Transgenic

Plant cell cultures

Bioreactors

Bioprocess

Optimization

ABSTRACT

A review of over 15 years of research, development and commercialization of plant cell suspension culture as a bioproduction platform is presented. Plant cell suspension culture production of recombinant products offers a number of advantages over traditional microbial and/or mammalian host systems such as their intrinsic safety, cost-effective bioprocessing, and the capacity for protein post-translational modifications. Recently significant progress has been made in understanding the bottlenecks in recombinant protein expression using plant cells, including advances in plant genetic engineering for efficient transgene expression and minimizing proteolytic degradation or loss of functionality of the product in cell culture medium. In this review article, the aspects of bioreactor design engineering to enable plant cell growth and production of valuable recombinant proteins is discussed, including unique characteristics and requirements of suspended plant cells, properties of recombinant proteins in a heterologous plant expression environment, bioreactor types, design criteria, and optimization strategies that have been successfully used, and examples of industrial applications.

© 2009 Elsevier B.V. All rights reserved.

Contents

1. Introduction	169
2. Recombinant protein production using transgenic plant cell cultures	170
2.1. Plant transformation: transient and stable gene expression	170
2.2. Transgene expression systems	170
2.3. Stability of recombinant proteins in a heterologous expression environment	173
3. Bioreactor engineering for plant cell suspension culture	174
3.1. Bioreactor types	174
3.1.1. Stirred-tank bioreactor	174
3.1.2. Pneumatic bioreactor	174
3.1.3. Wave bioreactor	174
3.1.4. Membrane bioreactor	176
3.1.5. Miniature (scaled-down) bioreactor	177
3.2. Bioreactor design considerations	177
3.2.1. Plant cell growth and oxygen demand	177
3.2.2. Aggregation and rheological properties of suspended plant cell cultures	177
3.2.3. Shear sensitivity of suspended plant cell cultures	178
3.2.4. Foaming and wall growth	178
4. Process considerations for optimization of plant cell bioreactor systems	179
4.1. Bioreactor operation considerations	179
4.2. Process monitoring	180
5. Industrial applications	180
6. Conclusions	181
References	181

* Corresponding author. Tel.: +1 530 752 8314; fax: +1 530 752 1031.

E-mail address: kamcdonald@ucdavis.edu (K.A. McDonald).

1. Introduction

Currently, the majority of recombinant proteins including human biopharmaceuticals are produced using traditional eukaryotic systems, such as mammalian cells (45%), particularly Chinese hamster ovary (CHO) cells (35%), yeast (15%) and insect cells (baculovirus system), or in bacterial systems (40%) [1]. The market for current and late stage (Phase III) biopharmaceutical protein products is \$50 billion (US) today and is estimated to be \$100 billion (US) in 2010 [2]. A wide diversity of host expression systems are in development for improving recombinant protein production [3–5], including genetic engineering of bacterial hosts [6] and human cell lines (e.g. PER.C6) through cell engineering approaches [7]. Recently, plant-based systems (whole plant and plant cell/tissue culture) have been investigated as a promising bioproduction platform of recombinant proteins, particularly human therapeutics (i.e. biopharmaceutical proteins) due to their intrinsic safety (they do not propagate mammalian viruses and pathogens), cost-effective bioprocessing that leads to lower production and downstream costs (due to inexpensive chemically defined, protein-free culture media), and the capability for post-translation modifications (able to produce glycoproteins and complex multimeric proteins, showing similarity to their native counterparts in terms of *N*-glycan structure compared to mammalian cells) [8,9]. Table 1 compares the production characteristics of various host expression systems for *in vitro* production of recombinant proteins [2–4,10,11].

In vitro aseptic suspension culture of plant cells, tissues or organs under controlled environmental conditions has been developed over the past 50 years, primarily for the production of valuable medicinal secondary metabolites such as shikonin and paclitaxel (Taxol) [11]. With the advances in understanding plant cell metabolism, molecular biology and physiology [9,12,13], plant cell cultures are now being considered as an alternative for the production of recombinant products [11,14]. Dedifferentiated cell aggregates (i.e. callus) generated from transgenic plants can be cultured in liquid media to establish transgenic plant cell suspension cultures. Unlike whole plant systems, suspended plant cell cultures can be grown under controlled conditions in bioreactors to obtain reproducible product yield [11,14], and also have reduced environmental concerns related to transgene migration

since the recombinant organisms are confined within the production facility [2,11]. More importantly, transgenic plant cell cultures in bioreactors have advantages over whole plants for sustained biopharmaceuticals production including (1) simplified purification particularly for products secreted into the extracellular medium (plant cells secrete only about 100–500 mg/L of total soluble proteins of culture medium); (2) consistency in product quality and homogeneity achievable under controlled environmental conditions; (3) ease of compliance with cGMP requirements; (4) elimination of the need for cultivation and manipulation of greenhouse or field grown plants which can be quite labor intensive; (5) ability to use inducible promoter systems; (6) reduced potential for endotoxin and mycotoxin contamination derived from the plant and soil source, and (7) minimal cell-to-cell communication resulting in the possible reduction of systemic post-transcriptional gene silencing (PTGS), which occurs via plasmodesmata as well as the vascular system in whole plants [15,16]. Consequently, plant cell cultures integrate the advantages of a plant host with those of microbial and mammalian cell *in vitro* culture systems for recombinant protein production.

The goals of development of plant cell suspension cultures for recombinant protein production are generally to maximize functional product titer (mg-Product/(L-broth) or % of total soluble protein), volumetric productivity (mg-Product/(L-broth-day)) and specific productivity (mg-Product/(g-Biomass-day)) through choice of host, selection of transgenic cell lines, media optimization, bioreactor operational strategy and bioprocess optimization. Bioreactor design and operation is critical for successfully developing large-scale production process using plant cell cultures. Because the applications of plant cell culture for recombinant protein production are largely dependent on the cell line used and the product of interest, the topics of this review article are generalized. In this article, we will focus on plant cell suspensions grown *in vitro* (not including hairy root, shooty teratoma and algae cultures) using simple sugars as the main carbon and energy source (not including photobioreactors). We review (1) transgene expression systems and properties of recombinant proteins in the heterologous expression environment; (2) the engineering aspects of bioreactor types and design criteria; (3) optimization of plant cell bioreactor operation, and (4) current industrial application examples.

Table 1

Features of host expression systems for *in vitro* recombinant protein production (modified from references in [2–4,10,11]).

Features	Expression systems				
	Bacterial (prokaryote)	Yeast (eukaryote)	Insect cells (eukaryote)	Mammalian cells (eukaryote)	Plant cells (eukaryote)
Expression level	0.5–5 g/L	0.1–2 g/L	0.1–2 g/L	1–3 g/L	0.01–0.2 g/L
Secretion	Periplasmic	Possible	Possible	Possible	Possible
Post-translational modification	None/incorrect	Capable	Capable	Capable	Capable
Glycosylation ability	Absence/incorrect	Incorrect; no terminal alpha-(1,3)-mannose	Partial-correct	Yes; typically human-like	Yes; no terminal galactose and sialic acid; contains xylose; altered fucose linkage
Protein folding accuracy	Refolding required; low	Refolding required; medium	Proper folding; medium	Correct folding; high	Proper folding; high
Protein homogeneity	Low	Medium	Medium	High	High
Multimeric protein assembly	No	No	Yes	Yes	Yes
Cell line stability	High	High	Medium	Low	Medium
Production timescale	Short	Short	Medium	Long	Medium
Scale-up capacity	High	High	Medium	Medium	Medium
Contaminant risk pathogens	Endotoxins	Endotoxins	Low risk	Virus, prions, oncogenic DNA	Very low risks
Therapeutic risk	Yes	Yes	Yes	Yes	Unknown
Process developed	Industrial scale	Industrial scale	Pilot scale	Industrial scale	Pilot scale
Purification cost	High	High	Medium	High	Low
Production cost estimation	Low (\$20–100/g)	Low (\$20–100/g)	Medium (\$50–200/g)	High (\$1000–10,000/g)	Low (\$50–100/g)
Products on market	Yes	Yes	Yes	Yes	No

2. Recombinant protein production using transgenic plant cell cultures

The first examples, reported in 1990, of heterologous protein production using transgenic plant cell suspension cultures, were recombinant human serum albumin [17] and chloramphenicol acetyltransferase [18]. Today there are more than 300 reports of plant-based production of therapeutic proteins (antibodies, single chain variable fragments (scFv), antigens, vaccines, hormones, growth factors and human blood proteins) under various stages of research and development [2]. A number of research groups have successfully developed plant cell culture bioreactor systems using tobacco, rice, soybean and tomato, etc. for the expression of various recombinant proteins (Table 2). Recently a recombinant animal vaccine product against Newcastle disease virus (NDV) produced in transgenic tobacco cell cultures by Dow Agrosciences was approved by the USDA in February 2006 [19]. This represents a landmark for developing plant cell culture as a bioproduction platform for production of therapeutic protein products. However, to date the product yield of recombinant protein using plant cell cultures is low compared to that of mammalian and microbial host cells (Tables 1 and 2). Thus, improving the yield and quality of recombinant proteins represents important goals for the development of plant cell culture-based expression systems. An ideal transgenic plant cell culture system for recombinant protein production possesses features including (1) high specific growth rate, (2) ease of genetic transformation, (3) high protein expression ability, (4) low endogenous proteolytic activity, (5) low production of secondary metabolites (such as phenolics and o-quinones that can react with amino acids of the recombinant protein through oxidation causing changes in protein physico-chemical, structural and biological properties and complicate purification processes) [20,21], (6) post-translational modification capability, (7) correct and uniform glycosylation pattern and proper protein folding, (8) small aggregates and good homogeneous dispersion in a bioreactor, and (9) long-term cell line genetic stability. In this section, transgene expression systems (vectors), properties of recombinant proteins in the heterologous expression environment and a variety of strategies to enhance product stability will be discussed.

2.1. Plant transformation: transient and stable gene expression

A recombinant protein can be expressed transiently or stably in plant cells. For transient expression, the gene of interest is expressed for several days to a couple of weeks (6–14 days depending on the recombinant protein, the host and the expression system) after it is delivered into plant cells. In this case, the gene of interest is not integrated into the genome of plant cells. For stable expression, the gene of interest is integrated into the genome of host cells and will be passed to subsequent generations [22]. Transient expression by the agroinfiltration method [23] or using plant viral vectors [24] is very useful for screening, research and small-scale production of recombinant proteins. Transient expression has frequently given higher productivity than stably transformed lines; the differences were attributed to the relative timing of the onset of PTGS compared to transgene expression [25] and position effect of transgene integration into the host plant's genome [26]. In transient expression, high levels of gene product may accumulate prior to the initiation of PTGS. Stable expression of a foreign gene involves the development of transgenic plants. Transgenic plant cell suspension cultures can be developed from callus generated from tissues of stably transformed plants using established plant tissue culture protocols. For large-scale production, a transgenic plant-based system may be a better choice, but will require longer time periods (months to years) for development and optimization of transformation, selection of an independent transformation event, and plant regeneration [9].

The method used for plant transformation will depend on the plant host species [22,27]. Most stable gene transformation methods involve nuclear transformation such as *Agrobacterium*-mediated transformation [28,29], particle bombardment [30] or electroporation of protoplasts [22]. On the other hand, however, genetic instability of the dedifferentiated cells have been shown to result in somaclonal variation (gene drift), a potential limiting factor in developing plant cell cultures for recombinant protein production [31]. Significant reductions in the product yield attributed to genetic instability, transgene loss, variation in growth rate, or other undesirable genetic change have been reported for transgenic plant cell cultures when maintained by subculturing [32–34]. Thus, cryopreservation of transgenic plant cell lines for long-term conservation has been studied to allow the maintenance of a stable production system [35,36].

2.2. Transgene expression systems

An efficient plant transgene expression system, consisting of a promoter, targeting signal peptide, codon usage optimization of target gene and transcription terminator, is essential for recombinant protein production. The choice of promoter system significantly influences the production yield by affecting the transcription rate of the target gene, and also influences the bioreactor operating strategy. Promoters can be divided into two categories: constitutive and inducible promoters. Table 3 summarizes the features of various promoters that have been used in transgenic plants cells for recombinant protein production [37–41]. Constitutive promoters directly drive the expression of the target gene in all tissues and are largely independent of developmental factors. An example is the well-known *Cauliflower mosaic virus* (CaMV 35S) promoter. The target protein expressed by a constitutive promoter is considered to be a growth-associated product and is continuously produced until cells reach the stationary phase. Thus, the constitutive expression of a recombinant protein could result in an additional metabolic burden during plant cell growth and hence reduce the specific growth rate. The characteristics of the recombinant protein might also impact the plant cells' physiology due to intrinsic toxic properties of the product on the host cells or it may interfere with host cell metabolism.

One molecular approach to achieve high-level heterologous protein production in plant cells is to utilize inducible transgene expression systems [37–41]. Inducible promoters are modulated by the presence of specific external factors or compounds, such as light, temperature, or the concentrations of metal ions, alcohols, steroids and herbicides, etc. Such regulated expression systems are advantageous because they allow the cell growth and protein production phases to be independently optimized. This approach is particularly attractive when product synthesis is deleterious to cell growth and/or cell viability. Furthermore, since expression of a foreign gene linked to an inducible promoter can be induced at a specific stage during the host cell growth cycle, there is less potential for PTGS found in gene expression systems that use constitutive promoters in transgenic plants [42,43]. Recently, a chemically inducible, estrogen receptor-based (XVE) promoter system has been developed for regulating transgene expression in transgenic plants [44]. The inducible XVE promoter system is activated by estradiol, allowing tightly regulated transcription of target genes, and resulted in an eightfold increase in GFP expression over the constitutive CaMV 35S promoter in transgenic *Arabidopsis* and tobacco plants [44]. Other chemically inducible promoter systems have been developed in plant cells such as ethanol- [45] and dexamethasone- [46] inducible promoter systems.

Plant viral vectors, designed to increase the target gene copy number by the action of the viral replicase (with the gene of interest typically replacing the gene coding for the viral coat pro-

Table 2
Selected examples of recombinant protein production in plant cell suspension cultures.

Recombinant protein	Expression host cells	Promoter system	Localization	Bioreactor operation and culture conditions	Production level	Ref.
Human therapeutic proteins Human serum albumin (HSA)	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, suspension batch cultures	0.25 µg/L	[17]
	<i>O. sativa</i> (rice)	RAmy3D: inducible (sugar starvation)	Secreted, extracellular	2.5 L bubble column (BC), 27 °C, dark, 4 SLPM (2 vvm), two-stage culture	15 mg/L (flask); 77 mg/L (BC)	[173]
	<i>N. tabacum</i> cv BY-2 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, suspension batch cultures	11.5% of TSP	[174]
	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Flask, 120 rpm, 25 °C, dark, two-stage batch, 10 days	1 µg/(g-FCW)	[41]
Human erythropoietin	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Flask, 115 rpm, 28 °C, dark, two-stage batch, 10 days	150–200 mg/L, 20% of TSP	[106]
Human alpha-1-antitrypsin (AAT)	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Stirred-tank bioreactor, single pitched-blade impeller, 75 rpm, two-stage culture, 27 °C, DO controlled at 70%	75 mg/L, 5 mg/(g-DCW)	[105]
hGM-CSF (human granulocyte-macrophage colony stimulating factor)	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Cyclical semi-continuous culture (multiple growth and production phases) in a STR, 75 rpm, 27 °C, DO at 70%, 0.1–0.2 vvm	40–50 mg/L, 4–7 mg/(L-day), 1–4 mg/(g-DCW-day);	[144]
	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Membrane bioreactor, 25 °C, 130 rpm, two stage culture	40–110 mg/L, 3–12 mg/(L-day), 4–8 mg/(g-DCW-day)	[144]
	<i>N. benthamiana</i> (tobacco)	CaMV 35S	Secreted, extracellular	Stirred-tank bioreactor, 25 °C, single-pitched blade impeller, 50 rpm, DO controlled at 40% pH controlled at 6.4 during late growth phase, batch	100–247 mg/L, 4–10% of TSP	[99]
	<i>N. benthamiana</i> (tobacco)	Estadiol inducible XVE system	Secreted, extracellular	STR, 25 °C, batch, pitched blade impeller, 50 rpm, DO at 40%, pH at 6.4 during induction phase, 10 µM estradiol as inducer	<1 µg-FAAT/L (without pH control); 100 µg-FAAT/L (pH control);	[72]
	<i>N. benthamiana</i> (tobacco)	Cucumber mosaic virus inducible viral vector (CMViva)	Secreted, extracellular	STR, 25 °C, batch, pitched blade impeller, 50 rpm, DO at 40%, pH at 6.4 during induction phase, 10 µM estradiol as inducer	<1 µg-FAAT/L (without pH control); 60 µg-FAAT/L (pH control)	[72]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	STR, 25 °C, batch, pitched blade impeller, 50 rpm, DO at 40%, pH at 6.4 during induction phase, 10 µM estradiol as inducer	25 µg-FAAT/L (without pH control); 100 µg-FAAT/L (pH control)	[72]
	<i>N. tabacum</i> L. cv Havana SR (tobacco)	CaMV 35S	Secreted, extracellular	Flask, suspension batch cultures	250 µg/L	[175]
	<i>O. sativa</i> (rice)	RAmy3D: inducible	Secreted, extracellular	Flask, 110 rpm, 12 day, 25 °C, batch	105 µg/L	[176]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flasks, two stage batch cultures, 13 days	129 mg/L, 25% of TSP	[149]
	<i>N. tabacum</i> L. cv Havana SR (tobacco)	CaMV 35S	Secreted, extracellular	Encapsulation of suspended cells in alginate beads in a shake flask	Increased yield: 100–180 µg/L	[153]
Human lysozyme Human interleukin-2 Human interleukin-4 Human interleukin-12 Human alkaline phosphatase	<i>O. sativa</i> (rice)	RAmy3D	Intracellular	Stirred-tank bioreactor, batch, four-bladed hollowed-paddle impeller at 80–250 rpm, Perfusion rate in STR: 0.5, 1 and 2 day ⁻¹ for 16 days, 0.2 vvm, 25 °C	6.5 µg/L (flask); 7.6 µg/L (STR); 71.2 µg/L (STR in perfusion at 1 day ⁻¹)	[145]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Transgenic callus	3–4% of TSP	[177]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 150 rpm, 29 °C, batch	10 µg/L (E), 75 µg/L (I)	[178]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 150 rpm, 29 °C, batch	180 µg/L (E), 275 µg/L (I)	[178]
	<i>N. tabacum</i> cv Havana	CaMV 35S	Secreted, extracellular	Flask, 100 rpm, 25 °C, batch	60 µg/L (I), 175 µg/L (E)	[179]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 120 rpm, 27 °C, dark, batch	800 µg/L (E) (with gelatin)	[180]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 150 rpm, 28 °C, dark, batch	27 mg/L (without PVP);	[181]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 125 rpm, 25 °C, dark, batch	30 mg/L	[182]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 120 rpm, 27 °C, dark, batch	38 µg/L	[182]
	<i>N. tabacum</i> cv NT-1, <i>Glycine max</i> (soybean)	Chimeric (ocs) ₃ mas	Intracellular	Flask, 120 rpm, 27 °C, dark, batch	2 mg/L (I) (tobacco), 22 mg/L (I) (soybean)	[183]
Antigen Hepatitis B surface antigen (HBsAg)	<i>Glycine max</i> (soybean)	<i>Arabidopsis</i> ubiquitin	Intracellular, ER-retention	Flask, batch, 80 rpm, 16 h light/8 h dark	700 ng/(g-FCW)	[184]

Table 2 (Continued)

Recombinant protein	Expression host cells	Promoter system	Localization	Bioreactor operation and culture conditions	Production level	Ref.
<i>scFv</i> (single chain variable fragment)						
Anti-phytochrome single-chain Fv	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 120 rpm, 26 °C, batch	0.5% of TSP	[185]
Anti-HBsAg single-chain Fv	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Apoplast targeting	Flask, 120 rpm, 26 °C, batch	5 mg/(kg-DCW), 1 mg/L	[186]
Anti-CEA antigen single-chain Fv	<i>O. sativa</i> (rice)	Maize ubiquitin	Apoplast targeting and ER-retention	Transformed callus	4 µg/(g-FCW),	[60]
Heavy chain mAb anti-Ars	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Stirred-tank bioreactor, six-blade disc impeller, batch, 25 °C	150 µg/L, 430 µg/L (with DMSO)	[187]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 150 rpm, 7 days, 29 °C; medium additive: PVP batch	170 µg/L (I), 10 µg/L (E), 360 µg/L (E) (with PVP)	[188]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Stirred-tank bioreactor, batch, six-blade disk impeller, 150 rpm, 0.1–0.9 vvm	10–350 µg/L	[109]
Antibody						
Human mAb against HBsAg	<i>N. tabacum</i> cv BY-2 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 26 °C, dark	0.2–0.6% of TSP	[189]
Mouse mAb IgG anti-TMV	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Flask, 120 rpm, 24 °C, batch, 16 h light/8 h dark	15–45 µg/(g-FCW)	[190]
	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Periodic harvesting using hydroxyapatite-resin (<i>in situ</i> product recovery) in a shaking flask	3.6 mg/L, 6.5% of TSP, 12% of TSP (with PVP)	[108]
Mouse IgG anti <i>Streptococcus</i> surface antigen	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Magnetic stirrer, batch, 2 L, 30 days operation	3.2 mg/L, 10.8 mg/L (with PVP)	[191]
Human anti-rabies virus mAb	<i>N. tabacum</i> cv Xanthi (tobacco)	CaMV 35S	Apoplast targeting and ER-retention	Flask, 100 rpm, 25 °C, 16 h light/8 h dark, disposable plastic bioreactor	30 µg/(g-DCW)	[192]
Reporter protein						
Carot invertase	<i>N. tabacum</i> cv NT-1 (tobacco)	CaMV 35S	Secreted, extracellular	Stirred-tank bioreactor, dual six-blade turbines, continuous culture at 0.2 day ⁻¹ , 10 L, 75 days	1400 U/L (I), 150 U/L (E)	[146]
GUS (beta-glucuronidase)	<i>N. tabacum</i> cv BY-2 (tobacco)	Heat shock (HSP18.2); heat inducible	Intracellular product	Two stage cultures, 25 °C for cell growth, 35–37 °C for induction phase	GUS activity: 30 µmol/(min-mg-protein)	[193]
	<i>N. tabacum</i> cv BY-2 (tobacco)	Tomato light inducible rbcS	Intracellular product	Two-stage cultures, dark during cell growth, light during induction phase	GUS activity: 7 nmol/(min-mg-protein)	[194]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Bean yellow dwarf virus (BeYDV) viral vector	Intracellular product	Flask, 200 rpm, 7 days, 27 °C	GUS activity: 15 nmol/(min-mg-protein)	[195]
	<i>N. tabacum</i> cv BY-2 (tobacco)	Methyl jasmonate inducible potato cathepsin D inhibitor (CDI)	Intracellular product	A Maxblend-type bioreactor, medium was continuously replaced with fresh medium by a spin filtration device, DO was controlled between 1 and 4 mg/L, 25 °C	GUS activity: 1.5 nmol/(min-mg-protein), total GUS activity: 5 µmol/L	[147]
	<i>N. tabacum</i> cv BY-2 (tobacco)	Glucocorticoid-inducible GVG promoter	Intracellular product	Flasks, batch, 100 rpm, dark, 25 °C, dexamethazone (DEX) as inducer (0–30 µM)	GUS activity: 8–10 nmol/(min-mg-protein)	[196]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Potato oxidative stress-inducible (POD)	Intracellular product	Flasks, batch, 130 rpm, dark, 26 °C	GUS activity: 5–14 nmol/(min-mg-protein)	[197]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Abscissic acid (ABA) inducible	Intracellular product	Flasks, batch, 130 rpm, 24 °C, ABA as inducer: 0–40 µM	GUS activity: 0.8 µmol/(min-mg-protein)	[40]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Tetracycline inducible	Intracellular product	Flasks, batch, 130 rpm, 24 °C, tetracycline as inducer: 0–40 µM	GUS activity: 320 µmol/(min-mg-protein)	[40]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Copper inducible	Intracellular product	Flasks, batch, 130 rpm, 24 °C, CuSO ₄ as inducer: 0–300 0–40 µM	GUS activity: 6 µmol/(min-mg-protein)	[40]
	<i>N. tabacum</i> cv NT-1 (tobacco)	Light inducible	Intracellular product	Flasks, batch, 130 rpm, 24 °C, light treatment: 0–53 W/m ²	GUS activity: 290 µmol/(min-mg-protein)	[40]
Acid phosphatase (APase)	<i>Anchusa officinalis</i>	APase- inducible by phosphate starvation	Extracellular product	Batch, 200 rpm, DO at 30% 0.3 vvm, 25 °C perfusion at 0.4 vvd (day ⁻¹), perfusion at 0.4 vvd (day ⁻¹) with cell culture bleeding	Batch: 100 U/L-day, Perfusion at 0.4 vvd: 300 U/L-day, perfusion at 0.4 vvd with cell bleeding: 490 U/L	[125]
GFP	<i>N. tabacum</i> cv BY-2 (tobacco)	Estradiol-inducible Tomato mosaic virus system	Intracellular product	Flask, 135 rpm, 7 days, 25 °C, estradiol as inducer: 0–10 µM	10% of TSP after 4 day induction	[49]

BC, bubble column; DO, dissolved oxygen; ER, endoplasmic reticulum; E, extracellular; I, intracellular; DCW, dried cell weight; FCW, fresh cell weight; Fv, functional human alpha-1-antitrypsin; vvm, volume of gas per volume of culture per minute.

Table 3

Characteristics of various promoters for expressing foreign genes in plant cells.

Promoter	Example	Characteristics
Constitutive promoter	CaMV 35S, maize ubiquitin	1. Commonly used in plants 2. High levels of expression 3. Potential problem of PTGS
Inducible promoter (chemically)	Steroid-regulated	1. Easy to use for induction by simply adding inducer 2. Inducer-dependent toxic effects 3. Low dose required in plant cell cultures
	Ethanol-regulated	1. Inducer is simple with low toxicity 2. Volatility may be a problem
	Tetracycline-regulated	1. Short half-life of antibiotics; toxic effect 2. High dose required for induction 3. Continuous addition required
Inducible promoter (metabolic)	Metabolite-regulated: rice alpha-amylase RAamy3D (sugar starvation)	1. Need to change medium (media exchange); two-stage cultures required 2. Affects cellular metabolism of host cells
Inducible promoter (physical)	Temperature-regulated	1. Triggers heat shock response related proteins 2. Influences cellular metabolism 3. Additional energy required
	Light-regulated	1. Influences cell growth and cellular metabolism 2. Additional energy required

tein), are alternatives for regulated expression of foreign genes [47–49]. Newer versions use *Agrobacterium*-mediated infiltration to introduce the viral amplicon into the host plant cell due to the efficiency of agroinfiltration. Several genetically modified viral vectors, which are based on *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV) and *Potato virus X* (PVX), have been applied in many plant-based systems for the production of recombinant proteins over the past decade [45,50–52]. Recently a TMV-based viral vector was developed by Icon Genetics, Inc. (Halle, Germany), which has been acquired by Bayer Innovation GmbH, for large-scale production of a flu vaccine made in nontransgenic tobacco plants using *Agrobacterium*-mediated infiltration [19]. However, plant viral vectors are prone to vector instability, leading to reduced replication of the viral vector possibility due to methylation [53] and PTGS [54,55]. To address these issues, inducible plant viral vectors have been developed as alternative expression system by integrating the advantages of inducible promoters and plant viral vectors [24,47,50]. Recently, our group has developed a novel CMV-inducible viral amplicon (CMViva) expression system, and demonstrated that it allows tightly regulated expression of foreign genes and good production of functional human proteins in non-transgenic plant tissues using transient agroinfiltration, as well as in transgenic plant cell cultures [56–58]. The CMViva system has the functional components of the tripartite CMV genome (RNA1, RNA2 and RNA3). The CMV RNA1 gene, which encodes an essential component of viral replicase is controlled by the chemically inducible, estradiol-activated XVE promoter, so recombinant viral amplicons are only produced intracellularly under induction conditions. Other examples of inducible plant viral vectors in suspension-cultured plant cells include the estradiol-inducible *Tomato Mosaic Virus* (ToMV) amplicon system expressing GFP in tobacco BY-2 cells by Dohi et al. [49] and the ethanol-inducible *Bean Yellow Dwarf Virus* (BeYDV) amplicon expressing Norwalk virus capsid protein (NVCP) in tobacco NT1 cells by Zhang and Mason [45].

In addition to developing efficient transgene expression systems, other molecular approaches [13] have been applied to enhance recombinant protein production in plant cells such as gene codon usage optimization [59], linking the KDEL signal peptide to retain the expressed protein in the endoplasmic reticulum (ER) [60–62], co-expression of gene silencing suppressors [57,63], and anti-proteinase gene expression [64–66].

2.3. Stability of recombinant proteins in a heterologous expression environment

Recombinant proteins expressed in plant cell cultures can be secreted to the extracellular culture medium through the plant secretory pathway or retained in an intracellular compartment such as endoplasmic reticulum, cytoplasm, or vacuole. From a bioprocessing standpoint, secreted products offer a number of advantages over intracellular products, particularly for plant cell suspension culture production systems. First, because plant cells grow as aggregates, cell/liquid separation can be accomplished relatively easily even using *in situ* gravity sedimentation. Second, plant cells do not secrete many proteins and since protein-free growth medium is used for cultivation, purification is easier due to the reduction in contaminating proteins. Finally, for secreted products the plant cells can be maintained and/or reused in the bioreactor for continuous or multiple production cycles, thereby minimizing the turnaround time associated with batch cultures and increasing the overall productivity. However, recombinant proteins secreted into the culture medium are commonly degraded by proteolytic enzymes that are produced during plant cell cultivation or resulting from cell death/lysis [67,68] and/or may be unstable in the simple environment of plant cell culture medium [14,69]. To improve the stability of secreted recombinant proteins, recent investigations [67,70,71] have used protease inhibitors or protein stabilization agents, such as gelatin, BSA and other low-value proteins (for protein-based stabilization), mannitol (to regulate the osmotic pressure of the medium to minimize cell lysis), PVP, PEG, Pluronic F-68 and other polymers (as stabilizing agents for protection of the protein product from denaturing agents liberated from the plant cells into the medium). Although these approaches have been shown to decrease the degradation or loss of the recombinant protein during plant cell culture through different mechanisms, protease inhibitors have short lifetimes and are expensive for application in large-scale production processes, and the use of stabilizing agents could exhibit negative effects on cell growth and increase downstream processing costs. To address these issues, Huang et al. [72] proposed a bioreactor strategy involving pH control for improving functional recombinant protein production in transgenic tobacco cell culture, which can enhance recombinant protein stability and minimize proteolysis effects derived from cell cultures, illustrating the importance

of optimizing bioreactor environmental conditions as an effective alternative to adding protease inhibitors or protein stabilizing agents in plant cell culture.

Other approaches have been proposed for reducing proteolytic effects on recombinant proteins including (1) co-expression of protease inhibitors hindering endogenous protease activities along the cell secretory pathway or released into the culture medium [64–66] and (2) the development of specific protease-deficient host cells [73]. However, co-expression of protease inhibitors or use of specific protease-deficient hosts may influence plant host physiology and cell growth. Therefore, the capability of various genetic and/or bioprocessing strategies for preventing or minimizing recombinant protein degradation in plant cell cultures has to be evaluated on a case-by-case basis.

Another critical determinant of the product quality is the proper post-translational modification of the recombinant protein product. Differences in the post-translational modification pattern and/or heterogeneity in the pattern, may limit the possible applications of plant-made recombinant proteins. Although protein folding, assembly and glycosylation are highly conserved between plants and animals (sharing similar mechanisms for proteins entering the secretory pathways: *N*-linked glycosylation in plants occurs in the ER and Golgi apparatus, *O*-linked glycosylation only occurs in the Golgi apparatus, and molecular chaperones in the ER help to fold the protein) [62], some differences in the capacity for post-translational modification have been observed between plant and animal cells [74]. Plant cells tend to attach α -(1,3)-fucose and β -(1,2)-xylose in the glycan of plant-made recombinant human glycoprotein, which are absent in animal cells [8,75]. Additionally, plant-made recombinant human glycoprotein generally lacks the terminal galactose and sialic acid residues, which have been found on many human glycoproteins [8]. These minor differences in the glycan structures of plant-derived recombinant human glycoproteins may impact the product inherent stability, biological activity and immunogenicity to human. Researchers have suggested strategies to overcome this problem by expressing “humanized” plant-made glycoproteins [76,77] as has been done in other host systems.

3. Bioreactor engineering for plant cell suspension culture

Significant progress has been made on improving recombinant protein production through genetic engineering approaches such as enhanced transgene transcription, post-transcription stability, proper mRNA processing and improved translation efficiency [13]. In this section, the bioreactor engineering aspects of plant cell suspension cultures including bioreactor types and bioreactor design considerations will be addressed.

3.1. Bioreactor types

Stirred-tank bioreactors, bubble columns and air-lift bioreactors (pneumatic type bioreactor) are commonly applied for microbial fermentation and mammalian cell cultures [78]. These bioreactors can be applied with minor modifications to plant cell suspension cultures for recombinant protein production [79]. Table 4 summarizes the advantages and disadvantages of various types of bioreactors. The relative advantages and selection criteria for various bioreactors need to be considered according to the specific recombinant protein product and characteristics of the host plant cell. General criteria for choosing a suitable bioreactor and bioreactor design should consider adequate oxygen mass transfer to cells, low shear stress to cells and adequate mass transfer (nutrient supply to cells and product and byproduct removal from cells).

3.1.1. Stirred-tank bioreactor

The main advantages of stirred-tank bioreactors are their flexibility and their ability to provide high volumetric mass transfer coefficients and a homogeneous environment enabling protein production to be controlled easily. For the stirred-tank bioreactor, the impeller system (mixing system) is the most important element. Rushton turbines (resulting in a radial flow pattern), which can provide complete solids and gas dispersion and sufficient oxygen mass transfer, is the most frequently employed for microbial fermentation. However, Rushton turbines induce high turbulence around the impeller region and exhibit higher specific power input (P/V) and energy dissipation rate (ϵ) than other impellers with axial flow patterns (such as marine impellers, helical ribbon impellers, paddle impellers, pitched-blade impellers, and centrifugal cell-lift impellers [80–84]), and may result in shear damage on plant cells even at the minimum agitation speed [83]. Doran [83] reported a theoretical engineering analysis of impeller type and geometry for plant cell culture in a 10 m³ bioreactor indicating that a pitched-blade turbine (axial flow pattern) with the upward-pumping mode offered advantages compared to Rushton turbines for solid suspension and for reducing shear stress to plant cells when the power input was restricted by cell damage considerations. However, studies also indicate that the oxygen mass transfer ability of an upward-pumping pitch-blade impeller was poor in highly viscous culture broth [80], compared with that of the same impeller operated in the downward-pumping mode [85]. Impeller systems exhibiting slow-moving axial flow patterns with low tip speed up to 2.5 m/s, such as the marine impeller, pitched blade and helical-ribbon impeller [86], are considered suitable for plant cell cultures.

3.1.2. Pneumatic bioreactor

A pneumatic bioreactor (bubble column, air-lift and modified air-lift) is a type of gas–liquid dispersion bioreactor consisting of a cylindrical vessel where compressed air or gas mixture is introduced at the bottom of the vessel through nozzles, perforated plates or a ring sparger, for aeration, mixing and fluid circulation, without moving mechanical parts. Air-lift or modified air-lift bioreactors [87,88] contain a draft tube (internal loop) or external loop, which have the following advantages: (1) preventing bubble coalescence by directing them in one direction; (2) enhancing oxygen mass transfer by increasing the number of bubbles; (3) distributing shear stress more evenly; and (4) promoting the cyclical movement of fluid resulting in shorter mixing times. For a high cell density culture of plant cells, however, the issues of inadequate oxygen mass transfer and poor fluid mixing leading to inhomogeneities in biomass, nutrient, oxygen and pH, and extensive foaming (resulting from extracellular polysaccharides, proteins, fatty acids, and high superficial gas velocity) may become limiting factors in pneumatic bioreactor operation [89,90]. On the other hand, the features of low capital and operational cost (no moving mechanical parts and sealing elements), ease of scale up and aseptic operation over long time periods, efficient fluid circulation using internal or external recirculation loops, and adequate oxygen mass transfer by increasing the gas bubble–liquid interfacial area [87,88] of pneumatic bioreactors can be advantageous for operation of plant cell cultures at moderate biomass concentrations [91]. The relatively low shear stress environment in pneumatic bioreactors is particularly desirable for shear-sensitive plant cells.

3.1.3. Wave bioreactor

Wave bioreactors, possessing the advantages of low-cost (based on disposable, single-use technology) and low shear environment, utilize a non-gas permeable sterile bag comprised of polyethylene plastic film which contains suspended cells and culture medium. To date, such wave bioreactors are characterized by rocking driven wave motion where the mass and heat transfer are regulated by

Table 4
Comparison of characteristics of bioreactors for plant cell suspension cultures.

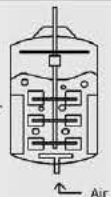
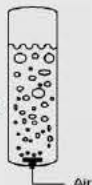
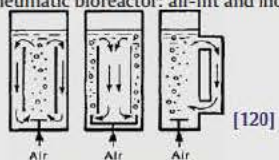
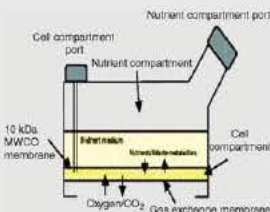
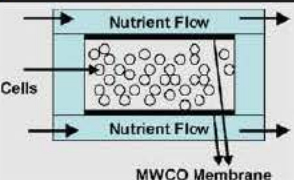
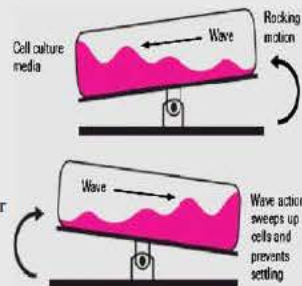
Bioreactor types	Features/advantages	Disadvantage
Stirred-tank bioreactor 	1. Commonly used 2. Ease of scale up 3. Useful for high viscously cell culture 4. High oxygen mass transfer ability 5. Good fluid mixing 6. Alternative impellers 7. Ease of compliance with cGMP requirements	1. High shear stress around the impeller 2. High capital and operational cost 3. Heat generation due to mechanical mixing 4. High energy cost due to mechanic agitation 5. Contamination risk with mechanical seal
Pneumatic bioreactor: bubble column 	1. Suitable for plant and animal cells 2. Easy to construct and scale up 3. Low operational cost 4. Low contamination risk 5. Low shear stress 6. No heat generation from mechanical agitation	1. Poor oxygen mass transfer ability 2. Poor fluid mixing in highly viscous cultures compared with stirred-tank bioreactor 3. Serious foaming under high aeration conditions
Pneumatic bioreactor: air-lift and modified air-lift  [Figure reprinted with permission of Springer, Inc.]	1. Suitable for plant and animal cells 2. Easy to construct and scale up 3. Low operational cost 4. Low contamination risk 5. Low shear stress 6. No heat generation from mechanical agitation 7. Multiple-choice of internal draft tubes 8. Better oxygen mass transfer than bubble column 9. Circulating flow pattern	1. Poor oxygen mass transfer ability compared with stirred-tank bioreactor 2. Poor fluid mixing for highly viscous culture compared with stirred-tank bioreactor 3. Serious foaming under high aeration conditions
Membrane bioreactor  Flat membrane [99] [Figure reprinted with permission of John Wiley & Sons, Inc.]	1. Disposable equipment 2. Ability to concentrate biomass and protein product in membrane compartment 3. Easy to withdraw extracellular product 4. Low shear stress 5. Low operational cost	1. Difficult to scale up 2. Oxygenation required 3. Low heat transfer rate 5. Difficult for on-line monitoring of culture conditions

Table 4 (Continued)

Bioreactor types	Features/advantages	Disadvantage
 Hollow Fiber Nutrient Flow Cells MWCO Membrane	6. Simplified medium exchange	
 Wave Bioreactor Cell culture media Wave Rocking motion Wave action sweeps up cells and prevents settling http://www.gelifesciences.com [Figure reprinted with permission of GE Healthcare, Inc.]	1. Disposable equipment 2. Low shear stress 3. High oxygen mass transfer 4. Useful for high cell density cell culture 5. Low operational cost 6. Reduce cleaning, in-house sterilization requirements 7. Increase flexibility 8. Light weight	1. Difficult to scale up for large-scale applications 2. Difficult to apply advanced cell culture operational strategies 3. Low heat transfer rate
 Miniature bioreactor www.applikon.com [Figure reprinted with permission of Applikon Biotechnology, Inc.]	1. Disposable equipment 2. High-throughput screening platform 3. Easy for heat transfer 4. Small volume 5. Research only	1. Difficult to scale up 2. Microenvironment can not exactly represent environment in large-scale process 3. Difficult for on-line monitoring of culture conditions

rocking rate, rocking angle and medium filling volume. While the wave bioreactor is rocking, the surface of the medium in the bag is continuously renewed and bubble-free surface aeration takes place resulting in oxygenation and bulk mixing with less shear stress to cultivated cells. WaveBiotech (www.wavebag.com), now part of GE Healthcare, is a leading company on the construction and application of the Wave Bioreactor™ and has the largest scale capability up to 500 L culture volume based on their hydrodynamic characterization. Researchers have demonstrated the potential application of the wave bioreactor for cultivating mammalian, tobacco, grape and apple suspension cells [92–94]. Eibl and Eibl achieved high plant cell (*V. vinifera*) biomass productivities of 40 g-FCW/(L-day) with a doubling time of 2 days and observed that there was no significant change in cell morphology when compared to cultivations in stirred-tank bioreactors [94]. Other advantages include time savings (the cleaning and sterilization processes are not needed), reduced foaming, easy operation and low risk of contamination (Table 4).

3.1.4. Membrane bioreactor

Membrane bioreactors (compartmentalized bioreactors), which utilize specialized membranes with specific molecular weight cut-off (MWCO) for either *in situ* aeration, nutrient supply, or product separation, are designed to retain cell biomass and also possibly recombinant protein product in a cell compartment. This type of bioreactor has been investigated primarily for mammalian and insect cell cultures [95–97]. Although, membrane bioreactors result in less shear stress to cultivated cells, studies indicated that mass transfer is an important consideration even for relatively slow growing plant cells [98]. McDonald et al. [99] applied a membrane bioreactor for the production of recombinant human alpha-1-antitrypsin (AAT) using transgenic rice cell culture with a rice alpha amylase promoter, Ramy3D, which is activated under sugar starvation conditions, resulting in extracellular product titer close to 250 mg/L and 4–10% of the extracellular total soluble protein. However, the membrane bioreactor is primarily used for small scale processes and is difficult to scale up for large-scale applications,

although it may be useful for certain smaller scale applications such as patient-specific therapeutics.

3.1.5. Miniature (scaled-down) bioreactor

Miniature bioreactor has been developed as a high-throughput screening platform for testing bioreactor operation conditions, selection and evaluation of recombinant cell lines, media optimization, and investigating important factors for scaling up a bioprocess and downstream process [100–102]. Miniature bioreactor platforms including shake flasks (25–1000 mL), microtiter plates (6, 12, 24, 96, with up to 384 wells and a few microliter to milliliter volumes), spin tubes (5–50 mL), and miniature stirred and bubble column bioreactor [100], which can mimic the micro- and macro-environments of large-scale bioproduction, have also been applied as a scale-down tool [103]. However, miniature bioreactors are less instrumented and also have limited opportunity for off-line sampling due to the small volumes used, suggesting that there is a gap between information available from the lab-scale, pilot-scale or commercial-scale bioreactor obtained by both on-line and off-line measurements. Therefore, miniature bioreactors may not be able to accurately mimic the large-scale process conditions (such as oxygen mass transfer, shear stress and fluid hydrodynamics).

3.2. Bioreactor design considerations

Bioreactor designs must provide an environment that is able to optimize the growth and productivity of the genetically engineered host cells ranging from shear sensitive and fragile cells (mammalian and some plant cells) to robust microorganism (bacteria and yeast). Plant cells exhibit attractive features for recombinant protein production (Table 1), however, their biological and physiological characteristics in suspension cultures, which are distinctive from bacterial and mammalian cells, might limit their application in large-scale production. Therefore, the morphological properties and cellular physiology of plant cells in suspension culture and the bioreactor engineering considerations including growth, nutrient uptake and production kinetics, oxygen and heat transfer, and fluid hydrodynamics need to be incorporated together to design an effective bioreactor. Important bioreactor design considerations for plant cell cultures are discussed in this section including: (1) plant cell growth and oxygen demand, (2) aggregation, rheological properties and shear sensitivity of plant cell cultures, and (3) foaming and wall growth.

3.2.1. Plant cell growth and oxygen demand

Plant cells grown in suspension culture generally exhibit a longer doubling time (20–100 h) than that of bacterial (30 min–1 h), yeast (2–3 h) and mammalian cells (24–48 h). Tobacco BY-2 cells have been shown to possess a higher growth rate and shorter doubling time (about 12 h) compared with other plant host species [80,104]. Other transgenic tobacco species (such as tobacco NT-1 or *Xanthi* cells) expressing a GUS (beta-glucuronidase) protein have a doubling time of ca. 1.5–2 days [79,80]. For other host cells, rice cell culture producing recombinant human AAT in a stirred-tank bioreactor has been reported to have a doubling time of 2 days [105] although a longer doubling time of 6 days has been observed in other studies [106].

In addition to the nutrients dissolved in the culture medium, oxygen is the most important gaseous substrate required for cellular growth and aerobic metabolism of suspended plant cells in *in vitro* culture. Oxygen uptake rate (OUR) can be used as an indicator for monitoring the physiology and oxygen demand of plant cells during suspension culture in a bioreactor. A typical OUR value for plant cells is about 5–10 mmol-O₂/(L-h), compared with 10–90 mmol-O₂/(L-h) for microbial cells and 0.05–10 mmol-O₂/(L-h) or $0.02\text{--}0.1 \times 10^{-9}$ mmol/(cell-h) for mammalian cells

(depending on the cell density and cell line type). The specific OUR (SOUR) is about 0.8 mmol-O₂/(g-DCW-h) for transgenic rice cell cultures expressing recombinant human alpha-1-antitrypsin [105] and 0.3–0.5 mmol-O₂/(g-DCW-h) for transgenic tobacco NT-1 cells expressing recombinant GUS protein [79]. Although the oxygen demand of plant cells for cell growth is relatively low because of their slow metabolism compared with microbial cells, the volumetric productivity for a high cell density plant cell culture is limited by inadequate oxygen mass transfer resulting from the high apparent viscosity of the cell culture broth and dissolved oxygen concentration (DO) [81,107,108]. Inadequate oxygen mass transfer in a bioreactor has been demonstrated to inhibit transgenic tobacco cell growth and reduce recombinant antibody heavy chain production [108,109]. Gao and Lee [79] studied the effect of oxygen supply on genetically modified tobacco cells and found that an increase in the oxygen supply enhanced specific growth rate, maximum cell concentration, consumption rate of glucose and fructose and GUS protein production yield in shake flask, stirred-tank and air-lift bioreactors. In addition, dissolved oxygen concentration is also critical for maintaining cell growth and viability. Typically the critical dissolved oxygen concentration for plant cell cultures in a bioreactor has been reported as 1.3–1.6 g/m³, roughly corresponding to 20% of air saturation [110].

To meet a general oxygen uptake rate of 5–10 mmol-O₂/(L-h) for plant cell cultures, a typical volumetric oxygen mass transfer coefficient (k_La) value required in a bioreactor operation for plant cell cultures is between 10 and 50 h⁻¹ [111], which is lower than that for microbial fermentation (100–1000 h⁻¹) [112,113] and slightly higher than that of mammalian cell culture (0.25–10 h⁻¹). In addition, aeration rates in the range of 0.05–0.1 vvm (volume of gas per volume of culture per minute) are typically used in a stirred-tank bioreactor and 0.5–1 vvm are applied in a pneumatic bioreactor. However, high aeration rates may result in severe foaming problems and gas-stripping effects (CO₂, ethylene or other volatile metabolites) which can inhibit plant cell growth [114,115], particularly for pneumatically agitated bioreactors. Thus, the design of an aeration system to generate as many small bubbles as possible (to increase the gas–liquid interfacial area), resulting in enhancing oxygen mass transfer, has been investigated [116]. Additionally, head-space gassing, aeration with oxygen-enriched air or increasing bioreactor pressure may be a convenient way to maintain dissolved oxygen concentration control under lower aeration rate conditions. On the other hand, although lower k_La often results in poor cell growth, higher k_La cannot guarantee good growth of plant cells. Early studies have suggested that plant cells grow better in suspension cultures at a restricted range of k_La [117,118].

3.2.2. Aggregation and rheological properties of suspended plant cell cultures

Plant cells in suspension culture are derived from callus tissues and tend to form aggregates and large clumps due to the failure of the daughter cells to separate from the parent cells after division [119] and the secretion of extracellular polysaccharides may also contribute to increased cell adhesion [120]. Size distribution of aggregates is dependent on plant host species, method of inoculum preparation, cell growth stage, medium composition, bioreactor types and culture conditions. Formation of cell aggregates generally promotes cellular organization and differentiation resulting in improving secondary metabolite production, furthermore, it also impacts mass transfer and can lead to oxygen, nutrient or chemical inducer inhomogeneities inside large cell aggregates [80]. Under these conditions the inner cells of the aggregates may become nutrient and oxygen deficient, which may have adverse effects on plant cell growth and affect recombinant protein yield and quality. Although moderate cell aggregation (200–500 μm) is sometimes advantageous since it enhances sedimentation rates, facilitating

media exchange as well as *in situ* recovery of culture broth during downstream processing, generation of large cell aggregates (~1–2 mm) is undesirable since this complicates the bioreactor operation, enhances mass transfer limitations and makes cell aggregates more susceptible to hydrodynamic stress, resulting in cell damage, attributed to aggregate surface attrition [121] and aggregate shattering [122].

Rheological properties of plant cells during suspension culture are dependent on cell aggregate size and morphology, biomass concentration, cell growth stage and culture conditions. Plant cells tend to transition from spherical to elongated shapes when cell division is terminated [123]. Curtis and Emery [124] studied the rheological properties of 10 different plant cells in shake flasks and found that elongated plant cell morphology in tobacco (*Nicotiana tabacum*) batch culture exhibited a power-law type (with a power law index of 0.6) fluid rheological property, resulting in higher apparent viscosity, compared to spherical cells. On the other hand, semi-continuous tobacco cell culture displayed Newtonian rheological behavior and tobacco cells did not elongate when grown in semi-continuous culture (they remained nearly spherical in shape), confirming the dependence of rheology on plant cell morphology [124]. Furthermore, elongated plant cell morphology may lead to higher packed cell volume (PCV) at a given dried cell weight (DCW) concentration, attributed to a more loose cellular network under packed conditions [125]. Wagner and Vogelmann [126] observed that there was a morphology change from pellet culture to suspension culture containing mostly single cells in a scale up study from a shake flask to a bioreactor, indicating the importance of the actual culture environment.

Kato et al. [127] and Curtis and Emery [124] both found that culture spent media was not responsible for the overall broth viscosity and that the viscous and non-Newtonian fluid character of the culture was due primarily to the plant cell morphology (elongated and filamentous cells) and high biomass concentration (with dry cell weight over 10 g/L and PCV over 50–60%). Specifically, Kato et al. [127] indicated that the apparent viscosity of *N. tabacum* cell culture broth was increased by a factor of 27 throughout the batch culture period and the filtrate (cell-free broth) was only increased from 0.9 to 2.2 cP. A typical apparent viscosity of plant cell culture broth is 4–150 cP (0.004–0.15 N s/m²). Bingham fluid ($\tau = \tau_0 + K\dot{\gamma}^n$, where τ is shear stress, τ_0 is yield stress, K is consistency index, $\dot{\gamma}$ is shear rate, and n is flow behavior index) and power-law fluid ($\tau = K\dot{\gamma}^n$) models have been applied to describe plant cell suspension cultures [110].

3.2.3. Shear sensitivity of suspended plant cell cultures

A single plant cell (100–500 μm in length and 20–50 μm in diameter) is about 10–100 times larger in size than bacterial (<1 μm in diameter), fungal (<100 μm in length and 5–10 μm in diameter) and mammalian cells (10–100 μm in diameter) and thus are capable of withstanding tensile strain, however, suspended plant cells are considered sensitive to shear stress due to their large volume of intracellular vacuoles (up to 90% of cell volume) and a rigid, inflexible cellulose-based cell wall [128]. Thus, plant cells are more susceptible to shear stress during the late exponential growth and early stationary phases when the cells are of relatively large size and contain large vacuoles [126]. Numerous studies on the shear sensitivity of cultured plant cells to hydrodynamic environment have been comprehensively investigated (detailed in [80,121,122,128–132]) by either cultivating suspended cells under shear forces (regulated by changes of agitation speed and/or aeration rate) during the period of cultivation in bioreactors or exposing plant cells to well-defined, laminar or turbulent flow conditions in specific devices. However, the clear mechanism of hydrodynamic shear stress induced-damage to suspended plant cells is not well understood due to the diversity of cell lines, aggregate size distribution

and cell morphologies, cell wall composition and culture age. Studies have reported that the cellular response of plant cells to hydrodynamic stress, including changes in cell viability (cell growth rate or membrane integrity), release of intracellular components (proteins or metabolites), changes in metabolism (OUR, mitochondrial activity, ATP concentration, cell wall composition, increase of calcium ions in cytoplasm) and changes in cell morphology and aggregation patterns [121,129,133–135], are influenced by the intensity and the exposure duration of the cells to shear force.

Scale-up of a plant cell suspension culture from a shake flask to a bioreactor is usually accompanied by a reduction in cellular productivity commonly attributed to the hydrodynamic stress which is associated with agitation and/or aeration in bioreactors. For a stirred-tank bioreactor, shear stress generated by the impeller system reduces the mean plant cell (*Catharanthus roseus*) aggregate size (80–100 μm in the shake flask and 64–80 μm in the stirred-tank bioreactor) but can also have an adverse effect on plant cell growth and viability (reducing the number of larger size aggregates causing cells to break and die) [136]. In a rapid fluid circulation zone created by impellers, plant cells are subject to the higher shear stress region of the impeller and thus more shear-induced damage on cells is generated [83]. For shear-sensitive plant cell cultures, therefore, reducing the shear stress intensity by decreasing the agitation speed of the impeller is a general solution. However, this reduction of agitation speed can lead to inadequate mixing and may conflict with enhancing oxygen and heat transfer rates in a high apparent viscosity plant cell culture broth. Furthermore, at high biomass concentration, low agitation rates can also enhance the clumping of cells into cell aggregates of varying sizes. Thus, a straightforward concept of critical shear stress (using regrowth of cells as an indicator) above which cell viability may be lost can be applied for impeller design and a critical shear stress between 50 and 200 N/m² has been reported [133]. A well-discussed study analyzing mixing issues in mammalian cell culture and microbial fermentation was reviewed by Amanullah et al. [86]. In addition, hydrodynamic stress damage associated with bubble rupture, which has been shown to be important in mammalian and insect cell cultures, has been investigated and found to be insignificant in plant cell suspension cultures [80,128].

3.2.4. Foaming and wall growth

Foaming usually occurs during the exponential growth phase due to the secreted or released extracellular proteinaceous compounds, polysaccharides and fatty acids [137,138] and may become exacerbated by cell lysis during the stationary phase. The suspended cells and proteinaceous compounds (including recombinant protein product) tend to be entrapped in the foam layer. The cells entrapped in the foam layer are subjected to nutrient and oxygen deficiency, resulting in the reduction of suspended biomass and productivity. These cells may also release proteases and secondary metabolites, and can generate a thick layer that adheres to the reactor wall (wall growth), impeller shaft and the sensors, hindering and disturbing the flow pattern of culture fluid. Under extreme foaming conditions, the foam layer could migrate up the gas outlet port and clog the air venting filters, restricting gas flow and making the culture susceptible to contamination [138]. Approaches to reduce the foaming include (1) reducing agitation speed and aeration rate without significantly impacting mixing intensity and mass transfer rates [138], (2) adding antifoam reagents leading to a reduction of the surface tension of the culture broth [139], (3) applying surface aeration or bubble free-aeration [140], and (4) using a mechanical foam breaker or installing an impeller above the culture broth to serve as a mechanical foam breaker. The application of antifoam reagents, which are added directly to the medium prior to inoculation (0.01% of working volume) and/or as required during cultivation, is the most common method [138,141]. Although

Table 5

Bioreactor operation strategies for improving recombinant protein production using plant cell suspension cultures.

Recombinant protein	Driven by a constitutive expression system	Driven by an inducible expression system
Intracellular product	1. Increase cell growth rate 2. Increase cell biomass concentration 3. Prolong exponential phase 4. Batch, fed-batch at high cell density culture (single stage) may be applicable 5. Semi-continuous/continuous culture can be applied to avoid severe cell aggregation and surface adhesion	1. Two-stage cultures: cell growth and protein production phases can be independently optimized 2. Bioreactor operational mode and conditions depend on the type of inducible system used 3. Semi-continuous or perfusion at high cell density with a slower cell growth rate during protein production phase
Extracellular product	1. Increase cell growth rate 2. Increase cell biomass concentration 3. Prolong exponential phase 4. Batch, fed-batch, and perfusion high cell density culture with bleed stream may be applicable 5. Semi-continuous culture to avoid severe cell aggregation and surface adhesion 6. <i>In situ</i> protein recovery system 7. Medium additives for enhancing protein stability and preventing proteolytic effects derived from cell cultures	1. Two-stage cultures: cell growth and protein production phases can be independently optimized 2. Bioreactor operational mode and conditions depend on the type of inducible system used 3. Semi-continuous or perfusion at high cell density with a slower cell growth rate during protein production phase 4. <i>In situ</i> protein recovery system 5. Medium additives for enhancing protein stability and preventing proteolytic effects derived from cell cultures

there are no reports about the direct negative effects of addition of antifoam reagents on plant cell growth [16], the addition of antifoam to the plant cell cultures may reduce the $k_L a$ value in the bioreactor.

4. Process considerations for optimization of plant cell bioreactor systems

A number of cell culture operational variables, which influence recombinant protein productivity and quality, and cell growth, need to be monitored, optimized and controlled for a plant cell suspension bioreactor process. Because the approaches depend strongly on the host plant cell line and the product of interest, the engineering strategies and considerations for optimization of bioreactor operation are generalized in this section.

4.1. Bioreactor operation considerations

The advanced cell culture operations developed for mammalian and microbial systems can be applied for plant cell suspension cultures such as batch, fed-batch, repeated-batch, two-stage culture, perfusion culture, semi-continuous and continuous culture [105,119,125,142–146] (Table 2). The choice of bioreactor operational modes, which support high product yield and cellular productivity, is highly dependent on the characteristics of plant host species (specific growth rate, tendency to form cell aggregates, foaming, and secondary metabolites production, etc.) and properties of the recombinant protein expression system, location and innate stability (e.g. driven by a constitutive or inducible expression system, secreted or intracellular product, etc.). Table 5 summarizes the potential bioreactor operation strategies for improving recombinant protein production using plant cell culture.

For production of a growth-associated recombinant protein (secreted or intracellular product) driven by constitutive promoter, protein productivity can be improved by increasing cell growth rate and biomass concentration (high cell density) and prolonging the exponential active cell growth phase. Fed-batch cultures have been applied to achieve high cell density culture when utilizing an effective substrate feeding strategy [147]. However, the accumulation of toxic or inhibitory metabolites in fed-batch cultures might limit recombinant protein productivity. Thus, perfusion culture with a

cell retention device can be an alternative to obtain high cell density culture and continuously withdraw cultured medium as well [125,143]. On the other hand, the cell growth rate in a batch or fed-batch plant cell suspension culture is usually retarded when the PCV reaches about 60–70% (high biomass volume fraction), resulting in the reduction of cellular metabolic activity [148]. Therefore, although fed-batch and perfusion culture can be applied to increase biomass concentration, semi-continuous culture or perfusion culture with a bleed stream have been demonstrated to be more suitable for high cell density culture due to plant cell aggregation, surface adhesion, and the high apparent viscosity observed at high biomass concentration [125,137]. In addition, the specific growth rate can be regulated by adjusting the dilution rate in a semi-continuous/continuous cell culture or the bleed stream in a perfusion culture.

For recombinant protein production driven by an inducible promoter system, two-stage cultures are typically applied in a bioreactor to allow the cell growth and protein production phases to be independently optimized. The bioreactor operation and conditions for induction phase (protein production phase) are largely dependent on the type of inducible promoter systems used (Table 3). The sugar-starvation Ramy3D promoter system (metabolically regulated) has been investigated to express recombinant human proteins including human α -1-antitrypsin [41,106] and human granulocyte-macrophage colony stimulating factor (hGM-CSF) [149] in transgenic rice cell cultures. In these studies, a media exchange to a sugar-free medium or nutrient medium containing an alternative carbon source [150] for inducing recombinant protein production was applied at a suitable time in the growth phase. During the induction phase without carbon source supplementation, however, the rice cell viability was significantly decreased, resulting in increased protease activity. Thus, a cyclical semi-continuous process, which alternates between growth and production phases, has been proposed to reuse the transgenic rice cells for long-term operation [144]. For a chemically inducible system, the timing of induction (cell exponential phase or stationary phase) and concentration and manner of application of inducer (single, multiple or continuous induction) applied to plant cell cultures are important parameters for optimizing inducible plant cell culture processes. This will need to be investigated based on the nature of the inducer (inducer stability and toxicity) and plant host

species (cell growth rate and aggregates) for enhancing transgene expression. Higher inducer concentrations and multiple or continuous application may benefit high cell density operational modes. Semi-continuous/continuous or perfusion bioreactor operation at high cell density with slower specific growth rate for a prolonged protein production phase can be applied for inducible production of the recombinant protein and the secreted recombinant protein can be continuously harvested and recovered from the cell culture broth. Our results indicate that OUR is an important physiological parameter to determine the optimal timing of induction (TOI). In the case of a chemically inducible, estrogen receptor-based promoter (XVE) system in tobacco cell culture, the optimal TOI occurs at the maximum OUR which occurs at the end of the exponential phase in a stirred-tank bioreactor (unpublished results). We also developed the semi-continuous culture production of recombinant human antitrypsin (AAT) using a chemically inducible plant viral vector in transgenic tobacco cell culture, resulting in fivefold increase in volumetric protein productivity of biologically functional recombinant AAT compared with batch operation (unpublished results).

Additionally, the productivity of secreted recombinant proteins, which could be unstable and rapidly degraded in the culture medium, can be improved through (1) addition of medium additives to enhance product stability and prevent the product from proteolysis derived from proteases generated by host cells [67,68,151], (2) *in situ* protein recovery (either by adding resins into the medium or by circulating the culture broth through a chromatography column external to the bioreactor) [108,152], and (3) immobilization of plant cells, in which cells are immobilized or encapsulated into a suitable carrier or support matrix in a bioreactor [153–155], to facilitate rapid recovery of secreted products from the culture broth.

4.2. Process monitoring

Systematic qualitative and quantitative descriptions of plant cell culture processes by on-line and off-line process monitoring methods are important for bioreactor operation, control, optimization and developing nutrient feeding and induction strategies [156]. Currently, process monitoring for a plant cell culture process is mainly applied for detecting cell growth and related physiological status such as biomass concentration, nutrient consumption, OUR, carbon dioxide production rate (CPR), respiratory quotient (RQ), DO and pH. Approaches have been proposed for off-line or on-line estimation of cell concentration in plant cell cultures based on the changes on medium conductivity [157], osmolarity [158], dielectric properties [159,160], and culture turbidity [161,162]. McDonald et al. [163] applied a focused beam reflectance method (FBRM) developed by Lasentec Inc., Redmond, WA) to accurately characterize several plant suspension cultures (*Oryza sativa*, *Nicotiana benthamiana* and *Trichosanthes kirilowii*) including biomass concentration and aggregate size. Recently a multisensory array consisting of metal oxide semiconductor sensors and a carbon dioxide sensor was proposed for on-line monitoring of the off-gas stream from plant cell cultures to predict the biomass concentration through the application of pattern recognition approaches (principal component analysis and artificial neural networks) [164]. Another sensor approach using a fiber-optic probe for on-line detecting of NAD(P)H fluorescence in tobacco cell suspension culture has been proposed to correlate the fluorescence signal to the biomass concentration [165]. To rapidly observe the product production kinetics and cellular dynamic responses to recombinant protein production, the recombinant protein can be fused with GFP, allowing on-line monitoring of the recombinant protein production by measuring the GFP fluorescence in the cell culture [166]. Furthermore, Su et al. [161] recently applied the extended Kalman filter and on-line measure-

ment of GFP fluorescence to develop a model-based state observer, allowing accurately estimation of GFP concentration in cell culture. Studies using the extended Kalman filter [167] and artificial neural network [168] have developed software sensors for monitoring and estimating biomass concentration in plant cell culture. Flow cytometry is a powerful technique for detecting the properties of cells or cellular particles in liquid suspension. Yanpaisan et al. [169] applied flow cytometry in a large-scale plant cell bioprocess for the measurement of nuclear DNA content, detecting cell cycle kinetics, cell counting and secondary metabolite accumulation.

5. Industrial applications

Plant cell culture provides an alternative bioproduction platform for animal and human therapeutics. In February 2006, USDA approved the first transgenic tobacco cell culture-produced recombinant glycoprotein protein, a veterinary vaccine based on the HN antigen (hemagglutinin/neuraminidase) derived from immunoprotective particles of NDV, for preventing avian NDV disease (developed by Dow AgroSciences (www.dowagro.com)) [19]. Other successful examples for the production of immunogenic proteins by Dow AgroSciences include the HA antigen (hemagglutinin protein) of avian influenza virus (AIV) and the VP2 structural protein of infectious bursal diseases virus (IBDV), which are driven by CaMV 35S or CsVMV (cassava vein mosaic virus) constitutive promoter in transgenic tobacco, potato or tomato cell cultures [170]. The recombinant immunoprotective proteins are produced and accumulated in the stationary phase of plant cell growth in cytoplasmic cell wall or membrane structure, and the product titer can be up to 4–30 mg/L [170,171].

Another exciting industrial example is by *Protalix Biotherapeutics* (www.protalix.com) in Israel involving the production of recombinant glucocerebrosidase (GCD) developed as a human therapeutic protein for people with Gaucher's disease, an inherited metabolic disorder that can lead to liver and bone problems. Currently, the cost for each patient is estimated to be \$200,000 a year on average for injection of a manufactured enzyme, making it become one of the most expensive drugs [19]. *Protalix Biotherapeutics* developed transgenic carrot cells in suspension culture for the production of recombinant glucocerebrosidase; production is driven by the CaMV 35S constitutive promoter and linked with a ER retention signal peptide [172] and this product is now in a Phase III clinical trial. *Protalix Biotherapeutics* has demonstrated that their plant-made recombinant GCD protein has identical catalytic kinetics characteristics compared with the mammalian-cell (CHO) produced enzyme (Cerezyme®). Interestingly, *Protalix Biotherapeutics* produces this recombinant glucocerebrosidase in large plastic bags instead of stainless steel vessels used for most mammalian and microbial systems.

Phyton Biotech (www.phytonbiotech.com) currently commercially produces paclitaxel compound (an anticancer secondary metabolite) using plant cell suspension cultures up to 75,000 L and has been a long-term supplier of this small molecule API Bristol-Myers Squibb's Taxol® oncology product. Recently, *Phyton Biotech* has turned to the production of recombinant proteins using transgenic plant cell suspension cultures and signed a development agreement with *Insmed* (www.insmed.com/), in which *Phyton Biotech* utilizes their cGMP plant cell culture technology to develop a manufacturing process that could meet the future demand of IPLEX™ (mecasermin rinfabate (rDNA origin) injection) as it expands with the development of IPLEX™ in broader therapeutic indications. IPLEX™ was approved by FDA in December 2005 for the treatment of growth failure in children with severe primary IGF-1 deficiency (Primary IGFD) or with growth hormone (GH) gene deletion. *Phyton Biotech* has made promising progress toward achieving a product yield of 2 g protein/(L-broth) [5].

In addition, several companies have been focusing on the production of therapeutic proteins in open-field-grown transgenic plants, including *Biolex Therapeutics* (interferon produced in duckweed against hepatitis C in Phase II trial), *Chlorogen* (TGF-beta produced by tobacco chloroplasts for treatment of ovarian cancer), *Cobento* (human intrinsic factor produced by *Arabidopsis* for treatment B-12 deficiency, approved in Ukraine), *Guardian Biotechnologies* (poultry vaccine produced by Canola against coccidiosis in Phase II trial in Canada), *Meristem Therapeutics* (lactoferrin produced by corn for gastrointestinal disorder treatment in Phase I trial; lipase produced by corn for treatment of cystic fibrosis disorders in Phase III trial), *Nexgen Biotechnologies* (poultry vaccine produced by tobacco against avian influenza (H5N1)), *Planet Biotechnology* (CaroRx by tobacco for tooth decay treatment in Phase II trial), *SemBioSys Genetics* (insulin produced by safflower for diabetes treatment in Phase II trial), and *Ventria Bioscience* (lysozyme produced by rice for diarrhea treatment in efficacy trial) [2,19].

Although the numerous studies have demonstrated the feasibility of developing plant cell culture for biopharmaceuticals production, only few examples have been commercially developed. Lower product yields (0.01–0.2 g/L) highlights the need to enhance productivity by a factor of at least 10–100, which is not unrealistic considering the impressive yield improvements in mammalian cell culture over the past 10 years.

6. Conclusions

Plant cell suspension culture as a bioproduction platform provides a number of unique advantages for recombinant protein production, especially human therapeutics, due to safety concerns and economics. Secreted recombinant protein products from plant cell cultures offer significant potential for reducing downstream processing (protein recovery and purification) cost. Various molecular and bioreactor engineering approaches proposed for improving recombinant protein yield and quality have been reviewed in this article. Future directions for improving recombinant protein yield and quality include: (1) developing a highly productive cell lines through cell engineering and system biology approaches; (2) enhancing secreted recombinant protein stability and preventing proteolytic degradation; (3) optimization of inducible plant cell culture systems and bioreactor operational strategies for maximizing cellular productivity; (4) investigations of gene silencing suppressors incorporated into plant cells for enhancing recombinant protein production; and (5) engineering humanized plant-made glycosylated proteins.

References

- [1] R.A. Rader, Expression systems for process and product improvement, *BioProcess* 6 (2008) 4–9.
- [2] J.L. Fox, Turning plants into protein factories, *Nature Biotechnology* 24 (2006) 1191–1193.
- [3] J.C. Yin, G.X. Li, X.F. Ren, G. Herrler, Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes, *Journal of Biotechnology* 127 (2007) 335–347.
- [4] L.M. Houdebine, Production of pharmaceutical proteins by transgenic animals, *Sang Thrombose Vaisseaux* 20 (2008) 43–50.
- [5] S. Lukjan, V. Srinivasan, G. Tous, S. Parekh, C. Swindell, M. Horn, Large-scale production of pharmaceutical proteins using plant cell suspension cultures, *In Vitro Cellular & Developmental Biology-Animal* 43 (2007) S50–S51.
- [6] H.P. Sorensen, K.K. Mortensen, Advanced genetic strategies for recombinant protein expression in *Escherichia coli*, *Journal of Biotechnology* 115 (2005) 113–128.
- [7] L.M. Barnes, A.J. Dickson, Mammalian cell factories for efficient and stable protein expression, *Current Opinion in Biotechnology* 17 (2006) 381–386.
- [8] V. Gomord, L. Faye, Posttranslational modification of therapeutic proteins in plants, *Current Opinion in Plant Biology* 7 (2004) 171–181.
- [9] R.M. Twyman, E. Stoger, S. Schillberg, P. Christou, R. Fischer, Molecular farming in plants: host systems and expression technology, *Trends in Biotechnology* 21 (2003) 570–578.
- [10] R. Boehm, Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms, *Annals of the New York Academy of Sciences* 1102 (2007) 121–134.
- [11] S. Hellwig, J. Drossard, R.M. Twyman, R. Fischer, Plant cell cultures for the production of recombinant proteins, *Nature Biotechnology* 22 (2004) 1415–1422.
- [12] S. Schillberg, R.M. Twyman, R. Fischer, Opportunities for recombinant antigen and antibody expression in transgenic plants – technology assessment, *Vaccine* 23 (2005) 1764–1769.
- [13] S.J. Streatfield, Approaches to achieve high-level heterologous protein production in plants, *Plant Biotechnology Journal* 5 (2007) 2–15.
- [14] E. James, J.M. Lee, The production of foreign proteins from genetically modified plant cells, *Advances in Biochemical Engineering/Biotechnology* 72 (2001) 127–156.
- [15] K.M. Crawford, P.C. Zambryski, Plasmodesmata signaling: many roles, sophisticated states, *Current Opinion in Plant Biology* 2 (1999) 382–387.
- [16] P.M. Doran, Foreign protein production in plant tissue cultures, *Current Opinion in Biotechnology* 11 (2000) 199–204.
- [17] P.C. Sijmons, B.M.M. Dekker, B. Schrammeijer, T.C. Verwoerd, P.J.M. Vandenelzen, A. Hoekema, Production of correctly processed human serum albumin in transgenic plants, *Bio-Technology* 8 (1990) 217–221.
- [18] R.S. Hogue, J.M. Lee, G.H. An, Production of a foreign protein product with genetically modified plant-cells, *Enzyme and Microbial Technology* 12 (1990) 533–538.
- [19] J. Travis, Is the drought over for pharming? *Science* 320 (2008) 473–477.
- [20] L. Jervis, W.S. Pierpoint, Purification technologies for plant-proteins, *Journal of Biotechnology* 11 (1989) 161–198.
- [21] H.M. Rawel, J. Kroll, S. Kulling, Effect of non-protein components on the degradability of proteins, *Biotechnology Advances* 25 (2007) 611–613.
- [22] K.K. Sharma, P. Bhatnagar-Mathur, T.A. Thorpe, Genetic transformation technology: status and problems, *In Vitro Cellular & Developmental Biology-Plant* 41 (2005) 102–112.
- [23] J. Kapila, R. DeRycke, M. VanMontagu, G. Angenon, An *Agrobacterium*-mediated transient gene expression system for intact leaves (vol 122, pg 101, 1997), *Plant Science* 124 (1997) 227–227.
- [24] Y. Gleba, V. Klimyuk, S. Marillonnet, Viral vectors for the expression of proteins in plants, *Current Opinion in Biotechnology* 18 (2007) 134–141.
- [25] T. Wroblewski, A. Tomczak, R. Michelmore, Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*, *Plant Biotechnology Journal* 3 (2005) 259–273.
- [26] S. Kumar, M. Fladung, Controlling transgene integration in plants, *Trends in Plant Science* 6 (2001) 155–159.
- [27] G. Hansen, M.S. Wright, Recent advances in the transformation of plants, *Trends in Plant Science* 4 (1999) 226–231.
- [28] R.B. Horsch, J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers, R.T. Fraley, A simple and general-method for transferring genes into plants, *Science* 227 (1985) 1229–1231.
- [29] A.K. Shrawat, H. Lorz, *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers, *Plant Biotechnology Journal* 4 (2006) 575–603.
- [30] T.M. Klein, E.C. Harper, Z. Svab, J.C. Sanford, M.E. Fromm, P. Maliga, Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process, *Proceedings of the National Academy of Sciences of the United States of America* 85 (1988) 8502–8505.
- [31] R. Offringa, M.J. de Groot, H.J. Haagsman, M.P. Does, P.J. van den Elzen, P.J. Hooykaas, Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation, *The EMBO Journal* 9 (1990) 3077–3084.
- [32] J.W. Gao, J.M. Lee, G.H. An, The stability of foreign protein-production in genetically modified plant-cells, *Plant Cell Reports* 10 (1991) 533–536.
- [33] P. Lambe, M. Dinant, R.F. Matagne, Differential long-term expression and methylation of the hygromycin phosphotransferase (Hph) and beta-glucuronidase (Gus) genes in transgenic pearl-millet (*Pennisetum-Glaucum*) callus, *Plant Science* 108 (1995) 51–62.
- [34] H.M. Vandermaas, E.R. Dejong, S. Rueb, L.A.M. Hensgens, F.A. Krens, Stable transformation and long-term expression of the gusa reporter gene in callus lines of perennial ryegrass (*Lolium-Perenne* L), *Plant Molecular Biology* 24 (1994) 401–405.
- [35] K. Schmale, T. Rademacher, R. Fischer, S. Hellwig, Towards industrial usefulness – cryo-cell-banking of transgenic BY-2 cell cultures, *Journal of Biotechnology* 124 (2006) 302–311.
- [36] J.S. Cho, S.M. Hong, S.Y. Joo, J.S. Yoo, D.I. Kim, Cryopreservation of transgenic rice suspension cells producing recombinant hCTLA4lg, *Applied Microbiology and Biotechnology* 73 (2007) 1470–1476.
- [37] M. Padidam, Chemically regulated gene expression in plants, *Current Opinion in Plant Biology* 6 (2003) 169–177.
- [38] J.R. Zuo, N.H. Chua, Chemical-inducible systems for regulated expression of plant genes, *Current Opinion in Biotechnology* 11 (2000) 146–151.
- [39] W. Tang, X.Y. Luo, V. Samuels, Regulated gene expression with promoters responding to inducers, *Plant Science* 166 (2004) 827–834.
- [40] H. Boetti, L. Chevalier, L.A. Denmat, D. Thomas, B. Thomasset, Efficiency of physical (Light) or chemical (ABA, tetracycline, CuSO₄ or 2-CBSU)-stimulus-dependent gus gene expression in tobacco cell suspensions, *Biotechnology and Bioengineering* 64 (1999) 1–13.
- [41] J. Huang, T.D. Sutcliffe, L. Wu, S. Nandi, K. Bengel, M. Terashima, A.H. Ralston, W. Drohan, N. Huang, R.L. Rodriguez, Expression and purification of functional

- human alpha-1-antitrypsin from cultured plant cells, *Biotechnology Progress* 17 (2001) 126–133.
- [42] H. Vaucheret, C. Beclin, M. Fagard, Post-transcriptional gene silencing in plants, *Journal of Cell Science* 114 (2001) 3083–3091.
- [43] H. Vaucheret, M. Fagard, Transcriptional gene silencing in plants: targets, inducers and regulators, *Trends in Genetics* 17 (2001) 29–35.
- [44] J.R. Zuo, Q.W. Niu, N.H. Chua, An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants, *Plant Journal* 24 (2000) 265–273.
- [45] X.R. Zhang, H. Mason, Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures, *Biotechnology and Bioengineering* 93 (2006) 271–279.
- [46] M. Samalova, B. Brzobohaty, I. Moore, pOp6/LhGR: a stringently regulated and highly responsive dexamethasone-inducible gene expression system for tobacco, *Plant Journal* 41 (2005) 919–935.
- [47] M. Mori, N. Fujihara, K. Mise, I. Furusawa, Inducible high-level mRNA amplification system by viral replicase in transgenic plants, *Plant Journal* 27 (2001) 79–86.
- [48] M. Gils, R. Kandzia, S. Marillonnet, V. Klimyuk, Y. Gleba, High-yield production of authentic human growth hormone using a plant virus-based expression system, *Plant Biotechnology Journal* 3 (2005) 613–620.
- [49] K. Dohi, M. Nishikiori, A. Tamai, M. Ishikawa, T. Meshi, M. Mori, Inducible virus-mediated expression of a foreign protein in suspension-cultured plant cells, *Archives of Virology* 151 (2006) 1075–1084.
- [50] C. Lico, Q. Chen, L. Santi, Viral vectors for production of recombinant proteins in plants, *Journal of Cellular Physiology* 216 (2008) 366–377.
- [51] J.A. Lindbo, High-efficiency protein expression in plants from agroinfection-compatible Tobacco mosaic virus expression vectors, *BMC Biotechnology* 7 (2007) 52–62.
- [52] B. Wagner, H. Fuchs, F. Adhami, Y. Ma, O. Scheiner, H. Breiteneder, Plant virus expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*, *Methods* 32 (2004) 227–234.
- [53] R.J. Hayes, R.H.A. Coutts, K.W. Buck, Stability and expression of bacterial genes in replicating geminivirus vectors in plants, *Nucleic Acids Research* 17 (1989) 2391–2403.
- [54] S.M. Angell, D.C. Baulcombe, Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA, *EMBO Journal* 16 (1997) 3675–3684.
- [55] R.G. Atkinson, L.R.F. Bielecki, A.P. Gleave, B.J. Janssen, B.A.M. Morris, Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector, *Plant Journal* 15 (1998) 593–604.
- [56] M.R. Sudarshana, M.A. Plesha, S.L. Uratsu, B.W. Falk, A.M. Dandekar, T.K. Huang, K.A. McDonald, A chemically inducible cucumber mosaic virus amplicon system for expression of heterologous proteins in plant tissues, *Plant Biotechnology Journal* 4 (2006) 551–559.
- [57] M.A. Plesha, T.K. Huang, A.M. Dandekar, B.W. Falk, K.A. McDonald, High-level transient production of a heterologous protein in plants by optimizing induction of a chemically inducible viral amplicon expression system, *Biotechnology Progress* 23 (2007) 1277–1285.
- [58] T.K. Huang, M.A. Plesha, B.W. Falk, A.M. Dandekar, K.A. McDonald, Comparison of alternative expression systems for production of a functional recombinant human protein in transgenic tobacco cell suspension cultures. *Biotechnology Progress* (submitted).
- [59] B.C. Geyer, S.P. Fletcher, T.A. Griffin, M.J. Lopker, H. Soreq, T.S. Mor, Translational control of recombinant human acetylcholinesterase accumulation in plants, *BMC Biotechnology* 7 (2007) 27.
- [60] E. Torres, C. Vaquero, L. Nicholson, M. Sack, E. Stoger, J. Drossard, P. Christou, R. Fischer, R. Fischer, Y. Perrin, Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies, *Transgenic Research* 8 (1999) 441–449.
- [61] H. Yasuda, Y. Hayashi, T. Jomori, F. Takaiwa, The correlation between expression and localization of a foreign gene product in rice endosperm, *Plant and Cell Physiology* 47 (2006) 756–763.
- [62] L. Faye, A. Boulaflois, M. Benchabane, W. Gomord, D. Michaud, Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming, *Vaccine* 23 (2005) 1770–1778.
- [63] W. Johansen, R.C. Wilson, Viral suppressor proteins show varying abilities and effectiveness to suppress transgene-induced post-transcriptional gene silencing of endogenous chalcone synthase in transgenic *Arabidopsis*, *Plant Cell Reports* 27 (2008) 911–921.
- [64] S. Komarnytsky, N. Borisjuk, N. Yakoby, A. Garvey, I. Raskin, Cosecretion of protease inhibitor stabilizes antibodies produced by plant roots, *Plant Physiology* 141 (2006) 1185–1193.
- [65] T.G. Kim, H.M. Kim, H.J. Lee, Y.J. Shin, T.H. Kwon, N.J. Lee, Y.S. Jang, M.S. Yang, Reduced protease activity in transformed rice cell suspension cultures expressing a proteinase inhibitor, *Protein Expression and Purification* 53 (2007) 270–274.
- [66] N.S. Kim, T.G. Kim, O.H. Kim, E.M. Ko, Y.S. Jang, E.S. Jung, T.H. Kwon, M.S. Yang, Improvement of recombinant hGM-CSF production by suppression of cysteine proteinase gene expression using RNA interference in a transgenic rice culture, *Plant Molecular Biology* 68 (2008) 263–275.
- [67] P.M. Doran, Foreign protein degradation and instability in plants and plant tissue cultures, *Trends in Biotechnology* 24 (2006) 426–432.
- [68] P.M. Doran, Loss of secreted antibody from transgenic plant tissue cultures due to surface adsorption, *Journal of Biotechnology* 122 (2006) 39–54.
- [69] B.M.Y. Tsoi, P.M. Doran, Effect of medium properties and additives on antibody stability and accumulation in suspended plant cell cultures, *Biotechnology and Applied Biochemistry* 35 (2002) 171–180.
- [70] J.H. Lee, N.S. Kim, T.H. Kwon, M.S. Yang, Effects of osmotic pressure on production of recombinant human granulocyte-macrophage colony stimulating factor in plant cell suspension culture, *Enzyme and Microbial Technology* 30 (2002) 768–773.
- [71] R.G. Soderquist, J.M. Lee, Enhanced production of recombinant proteins from plant cells by the application of osmotic stress and protein stabilization, *Plant Cell Reports* 24 (2005) 127–132.
- [72] T.K. Huang, M.A. Plesha, B.W. Falk, A.M. Dandekar, K.A. McDonald, Bioreactor strategies for improving production yield and functionality of a recombinant human protein in transgenic tobacco cell cultures, *Biotechnology and Bioengineering* 102 (2009) 508–520.
- [73] A. Schiermeyer, H. Schinkel, S. Apel, R. Fischer, S. Schillberg, Production of *Desmodus rotundus* salivary plasminogen activator alpha1 (DSPalpha1) in tobacco is hampered by proteolysis, *Biotechnology and Bioengineering* 89 (2005) 848–858.
- [74] S.A. Brooks, Appropriate glycosylation of recombinant proteins for human use – implications of choice of expression system, *Molecular Biotechnology* 28 (2004) 241–255.
- [75] N. Sethuraman, T.A. Stadheim, Challenges in therapeutic glycoprotein production, *Current Opinion in Biotechnology* 17 (2006) 341–346.
- [76] M. Schahs, R. Strasser, J. Stadlmann, R. Kunert, T. Rademacher, H. Steinkellner, Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern, *Plant Biotechnology Journal* 5 (2007) 657–663.
- [77] G. Nolke, R. Fischer, S. Schillberg, Production of therapeutic antibodies in plants, *Expert Opinion on Biological Therapy* 3 (2003) 1153–1162.
- [78] J.N. Warnock, M. Al-Rubeai, Bioreactor systems for the production of biopharmaceuticals from animal cells, *Biotechnology and Applied Biochemistry* 45 (2006) 1–12.
- [79] J. Gao, J.M. Lee, Effect of oxygen supply on the suspension culture of genetically modified tobacco cells, *Biotechnology Progress* 8 (1992) 285–290.
- [80] P.M. Kieran, Bioreactor design for plant cell suspension cultures, in: J.M.S.C.M.M.J. Tramper (Ed.), *Multiphase Bioreactor Design*, Taylor & Francis Ltd., Routledge, USA, 2001, pp. 391–426.
- [81] C.H. Ho, K.A. Henderson, G.L. Rorer, Cell-damage and oxygen mass-transfer during cultivation of *Nicotiana-Tabacum* in a stirred-tank bioreactor, *Biotechnology Progress* 11 (1995) 140–145.
- [82] F. Leckie, A.H. Scraggs, K.R. Cliffe, Effect of impeller design and speed on the large-scale cultivation of suspension-cultures of *Catharanthus-Roseus*, *Enzyme and Microbial Technology* 13 (1991) 801–810.
- [83] P.M. Doran, Design of mixing systems for plant cell suspensions in stirred reactors, *Biotechnology Progress* 15 (1999) 319–335.
- [84] J.J. Zhong, Z.W. Pan, Z.Y. Wang, J.Y. Wu, F. Chen, M. Takagi, T. Yoshida, Effect of mixing time on taxoid production using suspension cultures of *Taxus chinensis* in a centrifugal impeller bioreactor, *Journal of Bioscience and Bioengineering* 94 (2002) 244–250.
- [85] B.H. Junker, M. Stanik, C. Barna, P. Salmon, B.C. Buckland, Influence of impeller type on mass transfer in fermentation vessels, *Bioprocess Engineering* 19 (1998) 403–413.
- [86] B.C.B.A.W.N. Ashraf Amanullah, Mixing in the fermentation and cell culture industries, in: V.A.A.-O.S.M.K. Edward, L. Paul (Eds.), *Handbook of Industrial Mixing*, 2004, pp. 1071–1170.
- [87] P.M. Wang, T.K. Huang, H.P. Cheng, Y.H. Chien, W.T. Wu, A modified airlift reactor with high capabilities of liquid mixing and mass transfer, *Journal of Chemical Engineering of Japan* 35 (2002) 354–359.
- [88] T.K. Huang, P.M. Wang, W.T. Wu, Cultivation of *Bacillus thuringiensis* in an airlift reactor with wire mesh draft tubes, *Biochemical Engineering Journal* 7 (2001) 35–39.
- [89] H. Tanaka, Technological problems in cultivation of plant cells at high density (Reprinted from *Biotechnology and Bioengineering*, vol 23, pg 1203–1218, 1981), *Biotechnology and Bioengineering* 67 (2000) 775–790.
- [90] N.J. Smart, M.W. Fowler, An airlift column bioreactor suitable for large-scale cultivation of plant-cell suspensions, *Journal of Experimental Botany* 35 (1984) 531–537.
- [91] T.Y. Hsiao, F.T. Bacani, E.B. Carvalho, W.R. Curtis, Development of a low capital investment reactor system: application for plant cell suspension culture, *Biotechnology Progress* 15 (1999) 114–122.
- [92] V. Singh, Disposable bioreactor for cell culture using wave-induced agitation, *Cytotechnology* 30 (1999) 149–158.
- [93] B. Terrier, D. Courtois, N. Henault, A. Cuvier, M. Bastin, A. Aknin, J. Dubreuil, V. Petiard, Two new disposable bioreactors for plant cell culture: the wave and undertow bioreactor and the slug bubble bioreactor, *Biotechnology and Bioengineering* 96 (2007) 914–923.
- [94] R. Eibl, D. Eibl, Design and use of the wave bioreactor for plant cell culture, *Plant Tissue Culture Engineering* 6 (2006) 203–227.
- [95] W.W. Su, H.S. Caram, A.E. Humphrey, Optimal-design of the tubular micro-porous membrane aerator for shear-sensitive cell-cultures, *Biotechnology Progress* 8 (1992) 19–24.
- [96] V. Jager, A. Kobold, Propagation of *Spodoptera-Frugiperda* cells (sf9) and production of recombinant proteins with the baculovirus expression system using improved spinner flasks with membrane aeration, *Biotechnology Techniques* 9 (1995) 435–440.
- [97] H.N. Qi, C.T. Goudar, J.D. Michaels, H.J. Henzler, G.N. Jovanovic, K.B. Konstantinov, Experimental and theoretical analysis of tubular membrane aeration

- for mammalian cell bioreactors, *Biotechnology Progress* 19 (2003) 1183–1189.
- [98] G.F. Payne, N.N. Payne, M.L. Shuler, Bioreactor considerations for secondary metabolite production from plant-cell tissue-culture – indole alkaloids from *Catharanthus-Roseus*, *Biotechnology and Bioengineering* 31 (1988) 905–912.
- [99] K.A. McDonald, L.M. Hong, D.M. Trombly, Q. Xie, A.P. Jackman, Production of human alpha-1-antitrypsin from transgenic rice cell culture in a membrane bioreactor, *Biotechnology Progress* 21 (2005) 728–734.
- [100] J.I. Betts, F. Baganz, Miniature bioreactors: current practices and future opportunities, *Microbial Cell Factories* 5 (2006) 21–34.
- [101] N.K. Gill, M. Appleton, F. Baganz, G.J. Lye, Quantification of power consumption and oxygen transfer characteristics of a stirred miniature bioreactor for predictive fermentation scale-up, *Biotechnology and Bioengineering* 100 (2008) 1144–1155.
- [102] J.P. Diao, L. Young, P. Zhou, M.L. Shuler, An actively mixed mini-bioreactor for protein production from suspended animal cells, *Biotechnology and Bioengineering* 100 (2008) 72–81.
- [103] M. Micheletti, T. Barrett, S.D. Doig, F. Baganz, M.S. Levy, J.M. Woodley, G.J. Lye, Fluid mixing in shaken bioreactors: implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures, *Chemical Engineering Science* 61 (2006) 2939–2949.
- [104] A. Shinmyo, T. Shoji, E. Bando, S. Nagaya, Y. Nakai, K. Kato, M. Sekine, K. Yoshida, Metabolic engineering of cultured tobacco cells, *Biotechnology and Bioengineering* 58 (1998) 329–332.
- [105] M.M. Trexler, K.A. McDonald, A.P. Jackman, Bioreactor production of human alpha(1)-antitrypsin using metabolically regulated plant cell cultures, *Biotechnology Progress* 18 (2002) 501–508.
- [106] M. Terashima, Y. Murai, M. Kawamura, S. Nakanishi, T. Stoltz, L. Chen, W. Drohan, R.L. Rodriguez, S. Katoh, Production of functional human alpha(1)-antitrypsin by plant cell culture, *Applied Microbiology and Biotechnology* 52 (1999) 516–523.
- [107] J.E. Schlattmann, J.L. Vinke, H.J.G. Tenhoopen, J.J. Heijnen, Relation between dissolved-oxygen concentration and ajmalicine production-rate in high-density cultures of *Catharanthus-Roseus*, *Biotechnology and Bioengineering* 45 (1995) 435–439.
- [108] J.M. Sharp, P.M. Doran, Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures, *Biotechnology Progress* 17 (2001) 979–992.
- [109] F. Liu, J.M. Lee, Effect of culture conditions on monoclonal antibody production from genetically modified tobacco suspension cultures, *Biotechnology and Bioengineering* 4 (1999) 259–263.
- [110] P. Doran, Design of reactors for plant cells and organs, *Bioprocess Design and Control* 48 (1993) 115–168.
- [111] W.R. Curtis, A.L. Tuerk, Oxygen transport in plant tissue culture systems, *Plant Tissue Culture Engineering* 6 (2006) 173–186.
- [112] H. Yokoi, J. Koga, K. Yamamura, Y. Seike, H. Tanaka, High-density cultivation of plant-cells in a new aeration-agitation type fermenter, Maxblend fermenter(R), *Journal of Fermentation and Bioengineering* 75 (1993) 48–52.
- [113] F. Leckie, A.H. Scragg, K.C. Cliffe, An investigation into the role of initial *K_{la}* on the growth and alkaloid accumulation by cultures of *Catharanthus-Roseus*, *Biotechnology and Bioengineering* 37 (1991) 364–370.
- [114] N. Mirjalili, J.C. Linden, Gas-phase composition effects on suspension-cultures of *Taxus-Cuspidata*, *Biotechnology and Bioengineering* 48 (1995) 123–132.
- [115] A. Hohe, T. Winkelman, H.G. Schwenkel, CO₂ accumulation in bioreactor suspension cultures of *Cyclamen persicum* Mill. and its effect on cell growth and regeneration of somatic embryos, *Plant Cell Reports* 18 (1999) 863–867.
- [116] J. Lin, M.H. Han, T.F. Wang, T.W. Zhang, J.F. Wang, Y. Jin, Influence of the gas distributor on the local hydrodynamic behavior of an external loop airlift reactor, *Chemical Engineering Journal* 102 (2004) 51–59.
- [117] A. Kato, Y. Shimizu, S. Nagai, Biomass production of tobacco cells. 1. Effect of initial *K_{la}* on growth of tobacco cells in batch culture, *Journal of Fermentation Technology* 53 (1975) 744–751.
- [118] A. Pareilleux, R. Vinas, Influence of the aeration rate on the growth-yield in suspension-cultures of *Catharanthus-Roseus* (L.) G Don, *Journal of Fermentation Technology* 61 (1983) 429–433.
- [119] S. Chattopadhyay, S. Farkya, A.K. Srivastava, V.S. Bisaria, Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures, *Biotechnology and Bioengineering* 7 (2002) 138–149.
- [120] R.A. Taticek, M. Moo-Young, R.L. Legge, The scale-up of plant cell culture: engineering considerations, *Plant Cell, Tissue and Organ Culture* 24 (1991) 139–158.
- [121] P. Kieran, D. Malone, P. MacLoughlin, Effects of hydrodynamic and interfacial forces on plant cell suspension systems, Influence of Stress on Cell Growth and Product Formation 67 (2000) 139–177.
- [122] P.K. Namdev, E.H. Dunlop, Shear sensitivity of plant-cells in suspensions – present and future, *Applied Biochemistry and Biotechnology* 54 (1995) 109–131.
- [123] D.J. Cosgrove, Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement, *Plant Cell* 9 (1997) 1031–1041.
- [124] W.R. Curtis, A.H. Emery, Plant-cell suspension-culture rheology, *Biotechnology and Bioengineering* 42 (1993) 520–526.
- [125] W.W. Su, R. Arias, Continuous plant cell perfusion culture: bioreactor characterization and secreted enzyme production, *Journal of Bioscience and Bioengineering* 95 (2003) 13–20.
- [126] F. Wagner, H. Vogelmann, Cultivation of plant tissue culture in bioreactors and formation of secondary metabolites, in: W. Barz, E. Reinhard, M.H. Zenk (Eds.), *Plant Tissue Culture and its Biotechnological Application*, Springer, Berlin, 1977, pp. 245–252.
- [127] A. Kato, S. Kawazoe, Y. Soh, Biomass production of tobacco cells. 4. Viscosity of broth of tobacco cells in suspension culture, *Journal of Fermentation Technology* 56 (1978) 224–228.
- [128] E.H. Dunlop, P.K. Namdev, M.Z. Rosenberg, Effect of fluid shear forces on plant-cell suspensions, *Chemical Engineering Science* 49 (1994) 2263–2276.
- [129] J.J. Meijer, H.J.G. Tenhoopen, K.C.A.M. Luyben, K.R. Libbenga, Effects of hydrodynamic stress on cultured plant-cells – a literature survey, *Enzyme and Microbial Technology* 15 (1993) 234–238.
- [130] J.J. Meijer, H.J.G. Tenhoopen, Y.M. Vangameren, K.C.A.M. Luyben, K.R. Libbenga, Effects of hydrodynamic stress on the growth of plant-cells in batch and continuous-culture, *Enzyme and Microbial Technology* 16 (1994) 467–477.
- [131] P.F. MacLoughlin, D.M. Malone, J.T. Murtagh, P.M. Kieran, The effects of turbulent jet flows on plant cell suspension cultures, *Biotechnology and Bioengineering* 58 (1998) 595–604.
- [132] F.G. Camacho, A.C. Gomez, T.M. Sobczuk, E.M. Grima, Effects of mechanical and hydrodynamic stress in agitated, sparged cultures of *Porphyridium cruentum*, *Process Biochemistry* 35 (2000) 1045–1050.
- [133] P.M. Kieran, P.F. MacLoughlin, D.M. Malone, Plant cell suspension cultures: some engineering considerations, *Journal of Biotechnology* 59 (1997) 39–52.
- [134] T. Takeda, M. Seki, S. Furusaki, Hydrodynamic damage of cultured-cells of *Carthamus-Tinctorius* in a stirred-tank reactor, *Journal of Chemical Engineering of Japan* 27 (1994) 466–471.
- [135] T. Takeda, T. Kitagawa, Y. Takeuchi, M. Seki, S. Furusaki, Metabolic responses of plant cell culture to hydrodynamic stress, *Canadian Journal of Chemical Engineering* 76 (1998) 267–275.
- [136] H. Tanaka, H. Semba, T. Jitsufuchi, H. Harada, The effect of physical stress on plant-cells in suspension-cultures, *Biotechnology Letters* 10 (1988) 485–490.
- [137] W.W. Su, Bioprocessing technology for plant-cell suspension-cultures, *Applied Biochemistry and Biotechnology* 50 (1995) 189–230.
- [138] M.A. Abdullah, A.B. Ariff, M. Marziah, A.M. Ali, N.H. Lajis, Strategies to overcome foaming and wall-growth during the cultivation of *Morinda elliptica* cell suspension culture in a stirred-tank bioreactor, *Plant Cell Tissue and Organ Culture* 60 (2000) 205–212.
- [139] G.Q. Li, J.H. Shin, J.M. Lee, Mineral-oil addition as a means of foam control for plant-cell cultures in stirred-tank fermenters, *Biotechnology Techniques* 9 (1995) 713–718.
- [140] C. Bohme, M.B. Schroder, H. JungHeiliger, J. Lehmann, Plant cell suspension culture in a bench-scale fermenter with a newly designed membrane stirrer for bubble-free aeration, *Applied Microbiology and Biotechnology* 48 (1997) 149–154.
- [141] R. Wongsamuth, P.M. Doran, Foaming and cell flotation in suspended plant-cell cultures and the effect of chemical antifoams, *Biotechnology and Bioengineering* 44 (1994) 481–488.
- [142] L. Sajc, D. Grubisic, G. Vunjak-Novakovic, Bioreactors for plant engineering: an outlook for further research, *Biochemical Engineering Journal* 4 (2000) 89–99.
- [143] C. De Dobbeleer, M. Cloutier, M. Fouilland, R. Legros, M. Jolicoeur, A high-rate perfusion bioreactor for plant cells, *Biotechnology and Bioengineering* 95 (2006) 1126–1137.
- [144] M.M. Trexler, K.A. McDonald, A.P. Jackman, A cyclical semicontinuous process for production of human alpha(1)-antitrypsin using metabolically induced plant cell suspension cultures, *Biotechnology Progress* 21 (2005) 321–328.
- [145] S.Y. Lee, Y.H. Kim, Y.S. Roh, H.J. Myoung, K.Y. Lee, D.I. Kim, Bioreactor operation for transgenic *Nicotiana tabacum* cell cultures and continuous production of recombinant human granulocyte-macrophage colony-stimulating factor by perfusion culture, *Enzyme and Microbial Technology* 35 (2004) 663–671.
- [146] D.V. Des Molles, V. Gomord, M. Bastin, L. Faye, D. Courtois, Expression of a carrot invertase gene in tobacco suspension cells cultivated in batch and continuous culture conditions, *Journal of Bioscience and Bioengineering* 87 (1999) 302–306.
- [147] K.I. Suehara, S. Takao, K. Nakamura, N. Uozumi, T. Kobayashi, Optimal expression of GUS gene from methyl jasmonate-inducible promoter in high density culture of transformed tobacco cell line BY-2, *Journal of Fermentation and Bioengineering* 82 (1996) 51–55.
- [148] J.J. McCarthy, D. Ratcliffe, H.E. Street, The effect of nutrient medium composition on the growth-cycle of *Catharanthus-Roseus* G Don cells grown in batch culture, *Journal of Experimental Botany* 31 (1980) 1315–1326.
- [149] Y.J. Shin, S.Y. Hong, T.H. Kwon, Y.S. Jang, M.S. Yang, High level of expression of recombinant human granulocyte-macrophage colony stimulating factor in transgenic rice cell suspension culture, *Biotechnology and Bioengineering* 82 (2003) 778–783.
- [150] M. Terashima, Y. Ejiri, N. Hashikawa, H. Yoshida, Utilization of an alternative carbon source for efficient production of human alpha(1)-antitrypsin by genetically engineered rice cell culture, *Biotechnology Progress* 17 (2001) 403–406.
- [151] M. Benchabane, C. Goulet, D. Rivard, L. Faye, V. Gomord, D. Michaud, Preventing unintended proteolysis in plant protein biofactories, *Plant Biotechnology Journal* 6 (2008) 633–648.
- [152] E. James, D.R. Mills, J.M. Lee, Increased production and recovery of secreted foreign proteins from plant cell cultures using an affinity chromatography bioreactor, *Biochemical Engineering Journal* 12 (2002) 205–213.
- [153] T. Bodeutsch, E.A. James, J.M. Lee, The effect of immobilization on recombinant protein production in plant cell culture, *Plant Cell Reports* 20 (2001) 562–566.
- [154] F. Gilletta, C. Roisin, M.A. Fliniaux, A. Jacquin-Dubreuil, J.N. Barbotin, J.E. Nava-Saucedo, Immobilization of *Nicotiana tabacum* plant cell suspensions

- within calcium alginate gel beads for the production of enhanced amounts of scopolin, *Enzyme and Microbial Technology* 26 (2000) 229–234.
- [155] L. Osuna, E. Moyano, S. Mangas, M. Bonfill, R.M. Cusido, M.T. Pinol, A. Zamilpa, J. Tortoriello, J. Palazon, Immobilization of *Galphimia glauca* plant cell suspensions for the production of enhanced amounts of Galphimidine-B, *Planta Medica* 74 (2008) 94–99.
- [156] S. Zhang, J. Chu, Y. Zhuang, A multi-scale study of industrial fermentation processes and their optimization, *Bioengineering* 87 (2004) 97–150.
- [157] K.H. Kwok, P. Tsoulpha, P.M. Doran, Limitations associated with conductivity measurement for monitoring growth in plant-tissue culture, *Plant Cell Tissue and Organ Culture* 29 (1992) 93–99.
- [158] R. Madhusudhan, S.R. Rao, G.A. Ravishankar, Osmolarity as a measure of growth of plant-cells in suspension-cultures, *Enzyme and Microbial Technology* 17 (1995) 989–991.
- [159] J.J. Zhong, Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures, *Advances in Biochemical Engineering/Biotechnology* 72 (2001) 1–26.
- [160] G.H. Markx, C.L. Davey, D.B. Kell, P. Morris, The dielectric permittivity at radio frequencies and the bruggeman probe – novel techniques for the online determination of biomass concentrations in plant-cell cultures, *Journal of Biotechnology* 20 (1991) 279–290.
- [161] W.W. Su, B. Liu, W.B. Lu, N.S. Xu, G.C. Du, J.L. Tan, Observer-based online compensation of inner filter effect in monitoring fluorescence of GFP-expressing plant cell cultures, *Biotechnology and Bioengineering* 91 (2005) 213–226.
- [162] J.J. Zhong, K. Fujiyama, T. Seki, T. Yoshida, Online monitoring of cell concentration of *Perilla-frutescens* in a Bioreactor, *Biotechnology and Bioengineering* 42 (1993) 542–546.
- [163] K.A. McDonald, A.P. Jackman, S. Hurst, Characterization of plant suspension cultures using the focused beam reflectance technique, *Biotechnology Letters* 23 (2001) 317–324.
- [164] P. Komaraiiah, M. Navratil, M. Carlsson, P. Jeffers, M. Brodelius, P.E. Brodelius, P.M. Kieran, C.F. Mandenius, Growth behavior in plant cell cultures based on emissions detected by a multisensor array, *Biotechnology Progress* 20 (2004) 1245–1250.
- [165] K.J. Schlager, Instrumentation for plant health and growth, *Advances in Space Research* 14 (1994) 227–236.
- [166] W.W. Su, P. Guan, R.C. Bugos, High-level secretion of functional green fluorescent protein from transgenic tobacco cell cultures: characterization and sensing, *Biotechnology and Bioengineering* 85 (2004) 610–619.
- [167] J. Albiol, J. Robuste, C. Casas, M. Poch, Biomass estimation in plant-cell cultures using an extended kalman filter, *Biotechnology Progress* 9 (1993) 174–178.
- [168] J. Albiol, C. Campmajo, C. Casas, M. Poch, Biomass estimation in plant-cell cultures – a neural-network approach, *Biotechnology Progress* 11 (1995) 88–92.
- [169] W. Yanpaisan, N.J.C. King, P.M. Doran, Flow cytometry of plant cells with applications in large-scale bioprocessing, *Biotechnology Advances* 17 (1999) 3–27.
- [170] C.A. Mihaliak, M.J. Fanton, J.K. Mcmillen, Preparation of vaccine master cell lines using recombinant plant suspension cultures, United States Patent 20070107086 (2007).
- [171] T.J. Miller, M.J. Fanton, S.R. Webb, Stable immunoprophylactic and therapeutic compositions derived from transgenic plant cells and methods for production, United States Patent 20060222664 (2006).
- [172] Y. Shaaltiel, G. Baum, D. Bartfeld, S. Hashmueli, A. Lewkowicz, Production of high mannose proteins in plant culture, United States Patent 20080038232 (2008).
- [173] L.F. Huang, Y.K. Liu, C.A. Lu, S.L. Hsieh, S.M. Yu, Production of human serum albumin by sugar starvation induced promoter and rice cell culture, *Transgenic Research* 14 (2005) 569–581.
- [174] S. Matsumoto, K. Ikura, M. Ueda, R. Sasaki, Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells, *Plant Molecular Biology* 27 (1995) 1163–1172.
- [175] E.A. James, C. Wang, Z. Wang, R. Reeves, J.H. Shin, N.S. Magnuson, J.M. Lee, Production and characterization of biologically active human GM-CSF secreted by genetically modified plant cells, *Protein Expression and Purification* 19 (2000) 131–138.
- [176] S.Y. Hong, T.H. Kwon, J.H. Lee, Y.S. Jang, M.S. Yang, Production of biologically active hG-CSF by transgenic plant cell suspension culture, *Enzyme and Microbial Technology* 30 (2002) 763–767.
- [177] J.M. Huang, L.Y. Wu, D. Yalda, Y. Adkins, S.L. Kelleher, M. Crane, B. Lonnerdal, R.L. Rodriguez, N. Huang, Expression of functional recombinant human lysozyme in transgenic rice cell culture, *Transgenic Research* 11 (2002) 229–239.
- [178] N.S. Magnuson, P.M. Linzmaier, R. Reeves, G.H. An, K. HayGlass, J.M. Lee, Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture, *Protein Expression and Purification* 13 (1998) 45–52.
- [179] T.H. Kwon, J.E. Seo, J. Kim, J.H. Lee, Y.S. Jang, M.S. Yang, Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture, *Biotechnology and Bioengineering* 81 (2003) 870–875.
- [180] A. Becerra-Arteaga, H.S. Mason, M.L. Shuler, Production, secretion, and stability of human secreted alkaline phosphatase in tobacco NT1 cell suspension cultures, *Biotechnology Progress* 22 (2006) 1643–1649.
- [181] J.A. Francisco, S.L. Gawlak, M. Miller, J. Bathe, D. Russell, D. Chace, B. Mixan, L. Zhao, H.P. Fell, C.B. Siegall, Expression and characterization of bryodin 1 and a bryodin 1-based single-chain immunotoxin from tobacco cell culture, *Bioconjugate Chemistry* 8 (1997) 708–713.
- [182] P.C. Sehne, R.J. Ferl, Processing of preproinsulin in transgenic tobacco, *Protein Expression and Purification* 15 (1999) 188–195.
- [183] M.L. Smith, H.S. Mason, M.L. Shuler, Hepatitis B surface antigen (HBsAg) expression in plant cell culture: kinetics of antigen accumulation in batch culture and its intracellular form, *Biotechnology and Bioengineering* 80 (2002) 812–822.
- [184] T. Ganapathi, G. Sunil Kumar, L. Srinivas, C. Revathi, V. Bapat, Analysis of the limitations of hepatitis B surface antigen expression in soybean cell suspension cultures, *Plant Cell Reports* 26 (2007) 1575–1584.
- [185] S. Firek, J. Draper, M.R.L. Owen, A. Gandeche, B. Cockburn, G.C. Whitelam, Secretion of a functional single-chain fv protein in transgenic tobacco plants and cell-suspension cultures, *Plant Molecular Biology* 23 (1993) 861–870.
- [186] N. Ramirez, D. Lorenzo, D. Palenzuela, L. Herrera, M. Ayala, A. Fuentes, M. Perez, J. Gavilondo, P. Oramas, Single-chain antibody fragments specific to the hepatitis B surface antigen, produced in recombinant tobacco cell cultures, *Biotechnology Letters* 22 (2000) 1233–1236.
- [187] M.F. Wahl, G.H. An, J.M. Lee, Effects of dimethyl-sulfoxide on heavy-chain monoclonal-antibody production from plant-cell culture, *Biotechnology Letters* 17 (1995) 463–468.
- [188] N.S. Magnuson, P.M. Linzmaier, J.W. Gao, R. Reeves, G.H. An, J.M. Lee, Enhanced recovery of a secreted mammalian protein from suspension culture of genetically modified tobacco cells, *Protein Expression and Purification* 7 (1996) 220–228.
- [189] A. Yano, F. Maeda, M. Takekoshi, Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen, *Journal of Medical Virology* 73 (2004) 208–215.
- [190] R. Fischer, Y.C. Liao, J. Drossard, Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture, *Journal of Immunological Methods* 226 (1999) 1–10.
- [191] R. Wongsamuth, P.M. Doran, Production of monoclonal antibodies by tobacco hairy roots, *Biotechnology and Bioengineering* 54 (1997) 401–415.
- [192] L.S. Girard, M.J. Fabis, M. Bastin, D. Courtois, V. Petiard, H. Koprowski, Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture, *Biochemical and Biophysical Research Communications* 345 (2006) 602–607.
- [193] K. Yoshida, T. Kasai, M.R.C. Garcia, S. Sawada, T. Shoji, S. Shimizu, K. Yamazaki, Y. Komeda, A. Shinmyo, Heat-inducible expression system for a foreign gene in cultured tobacco cells using the HSP18.2 promoter of *Arabidopsis thaliana*, *Applied Microbiology and Biotechnology* 44 (1995) 466–472.
- [194] N. Uozumi, Y. Inoue, K. Yamazaki, T. Kobayashi, Light activation of expression associated with the tomato Rbcs promoter in transformed tobacco cell-line by-2, *Journal of Biotechnology* 36 (1994) 55–62.
- [195] T.S. Mor, Y.S. Moon, K.E. Palmer, H.S. Mason, Geminivirus vectors for high-level expression of foreign proteins in plant cells, *Biotechnology and Bioengineering* 81 (2003) 430–437.
- [196] Y. Nara, H. Kurata, M. Seki, K. Taira, Glucocorticoid-induced expression of a foreign gene by the GVG system in transformed tobacco BY-2 cells, *Biochemical Engineering Journal* 6 (2000) 185–191.
- [197] K.Y. Kim, S.Y. Kwon, H.S. Lee, Y. Hur, J.W. Bang, S.S. Kwak, A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells, *Plant Molecular Biology* 51 (2003) 831–838.