Chapter 3
Molecular Farming Using Bioreactor-Based Plant Cell Suspension Cultures for Recombinant Protein Production

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Abstract The need for biomanufacturing capacity for recombinant protein production to meet the expanding pharmaceutical and industrial market demands has gained increasing importance, leading to the development of new protein expression platforms capable of addressing requirements in terms of protein yield, product quality, and production cost. In the past few decades, molecular farming using plant cell-based expression systems (whole plants and *in vitro* plant cells, organ and tissue cultures) have been investigated as an alternative for the large-scale bioproduction of recombinant proteins. Molecular farming using bioreactor-based plant cell suspension cultures provides attractive features over recombinant microbial fermentation and mammalian cell cultures in terms of intrinsic safety, cost-effective biomanufacturing, and the capability for post-translation modifications. The current research and development, emerging techniques, commercialization and future prospects of molecular farming using bioreactor-based plant cell suspension cultures for production of recombinant proteins will be discussed in this chapter.

3.1 Introduction

The global market of recombinant protein products for pharmaceutical/therapeutic applications reached $86 billion in 2007 and is expected to increase to an estimated $160 billion in 2013, representing an annual growth rate of 11%. Large-scale biomanufacturing of recombinant protein products has received important considerations due to the expanding market demand with the additional applications of

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the industrial/technical recombinant proteins, resulting in new protein expression platforms and emerging technologies development such as molecular farming bioproduction platforms (transgenic animal and plant-based) in addition to mammalian cell cultures and microbial fermentation (Demain and Vaishnav 2009; Hacker et al. 2009).

Plant molecular farming refers to the large scale production of recombinant proteins, including plant-made pharmaceuticals (PMP) and plant-made industrial proteins (PMIP), in transformed plant cells or, transgenic plants. Plant-based molecular farming, including transgenic plants, transplastomic plants, transient expression by agroinfiltration in plants and plant tissues, virus-infected plants, plant tissue and organ cultures, and plant cell suspension cultures, have been investigated as an economical alternative bioproduction platform for recombinant protein production in the past 20 years (Obembe et al. 2011). Plant cell-based molecular farming offers attractive features over traditional microbe- and mammalian cell-based expression systems, including (1) their intrinsic safety (plant cells do not propagate mammalian viruses and pathogens and can be cultivated in animal-derived component free medium which are important considerations for therapeutics production), (2) cost-effective biomanufacturing leading to lower production costs (Shadwick and Doran 2005), and (3) the capability for protein post-translation modifications (such as the ability to produce glycoproteins with similarity to their native counterparts in terms of N-glycan structure compared to mammalian cells) (Gomord et al. 2010).

Plant cell suspension cultures grown in a fully contained bioreactor system offer additional features, compared to molecular farming using whole plants, for economical, sustained recombinant protein production, especially for biopharmaceuticals production, (Franconi et al. 2010; Hellwig et al. 2004) including (1) shorter biomanufacturing timescale of only few weeks required in plant cell culture process; (2) more consistency in product quality and homogeneity of the target protein N-glycan structures in controlled bioreactor operations (De Muynck et al. 2010; Lienard et al. 2007); (3) cost effective purification operations especially for secreted products (Rawel et al. 2007); (4) less contamination from endotoxin and mycotoxin; (5) safer production platform in a contained system, avoiding issues such as gene flow in the environment and potential contamination to the food chains (Franconi et al. 2010); and (6) ease of compliance with cGMP requirements (Shih and Doran 2009), etc. In this chapter, recent developments and future prospects of molecular farming using bioreactor-based plant cell suspension cultures (dedifferentiated plant cells such as tobacco, rice and carrot cell cultures, etc.) for recombinant protein production will be discussed.

3.2 Recombinant Protein Production Using Plant Cell Suspension Cultures

In vitro suspension cultured plant cells under controlled environmental conditions have been developed primarily for the production of valuable medicinal metabolites such as shikonin and paclitaxel (Taxol) in the past 50 years (Hellwig et al. 2004).

Currently various recombinant proteins for therapeutic (monoclonal antibodies, antigens, vaccines, hormones, growth factors and blood proteins, etc.), medical (e.g. gelatin and collagen for drug capsules), and industrial (enzymes such as cellulases, lignases and lipases for biofuel) applications can be expressed using plant cell cultures transformed with appropriate gene expression systems (Huang and McDonald 2009). In this section, the methods to establish transgenic plant cell suspension cultures will be presented.

3.2.1 Features of Plant Cell Suspension Cultures for Recombinant Protein Production

Molecular farming using bioreactor-based plant cell culture should exhibit the following features: (1) ease of genetic manipulation by stable transformation or transient expression, (2) high protein expression level, (3) low endogenous proteolytic activity, (4) high product stability (inside and outside of the cells), (5) low concentration of secondary metabolites, which may cause changes in expressed protein structural and biological properties and complicate downstream processes, (6) post-translational modification capability, uniform glycosylation pattern and proper protein folding, (7) small cell aggregates and good homogeneous dispersion in a bioreactor, (8) high specific growth rate, and (9) long-term cell line genetic and production stability, etc.

3.2.2 Method to Establish Transgenic Plant Cell Suspension Cultures

Transgenic plant cell suspension cultures developed for recombinant protein production are usually derived from stably transformed plant or plant tissues generated by mainly using *Agrobacterium*-mediated transformation methods (Offringa et al. 1990), although they can also be derived from initial transformed callus from an independent transformation event. Leaves, roots, shoots or petioles from grown plants can be used as initial explants. Callus cultures grown on agar containing appropriate plant growth regulating hormones initiated from explants from transgenic plants can be transferred to grow in a chemically defined media with continuous shaking to establish suspended cell cultures (Rao et al. 2009). After 5–14 days, the force from hydrodynamic/mechanical stress in continuous shaken or agitated liquid media results in a population of cell suspensions with healthy cells, dead and/or decaying cell material. The population of an initial suspended cells usually consists roughly of three fractions including (1) free single cells of various shapes, (2) clusters of 3–5 cells, small (up to 20 cells) and big cell aggregates (more than 20 cells), and (3) cell groups with a threadlike morphology, released from the callus. The outermost cell layers of the cell aggregates are removed due to the hydrodynamic force by the agitation of the shaker and then represent the fractions of single cells.

A dilution ratio of 1:1–1:5 (volume ratio of mother culture to fresh medium) is typically used to maintain the cell suspension culture. Fine cell fractions can be obtained and transferred using sterile pipette (2–10 mL). Sterile meshes with different sizes (such as Sigma cell dissociation kit) can be implemented to separate large aggregates and established a suspension culture only with single cells, clusters or small aggregates. A mesh size of 60 is used to initiate a suspension culture composed of big and small aggregates, clusters and single cells, size 80 is used to separate small aggregates, clusters and single cells, size 100 is used to obtain clusters and single cells, and size 150 is to isolate almost single cells. Observations using an inverted microscope can be used to confirm the formation of structures, single cell, clusters or cell divisions.

The preculture stage is carried out to propagate the single cells by inoculating the original cell population containing single cells and small cell aggregates in fresh nutrient media. The addition of plant growth regulators is usually required in the culture medium to promote rapid cell growth and maintain the cell morphology. After 1–2 weeks, part of the preculture (cells and nutrient medium) is transferred to other vessels or a bioreactor. Usually the cell suspension with some nutrient medium can be transferred from the smaller to the larger bioreactor to produce increasing amounts of cell suspension. The optimization of bioreactor operation for recombinant protein production will be discussed in later session. For some plant species, starting with a population of active single cells in a liquid medium, cell aggregates of various sizes will develop soon after initiation of growth, coexisting with some free suspended cells. The size distribution of cell aggregates and single cell morphology are important consideration for bioreactor optimization.

Subcultures are frequently conducted by a dilution of 1:5–1:10 with fresh nutrient medium at 1–2 week intervals to maintain suspended plant cells in a healthy and active condition for the establishment of long-term cell suspension cultures. The optimal dilution ratio (or inoculation ratio) and subculture frequency have to be experimentally determined for each individual species.

3.2.3 Example of Transgenic Nicotiana benthamiana Cell Suspension Cultures

3.2.3.1 Plant Cell Transformation and Media

Figure 3.1 shows the schematic presentation of the production of a recombinant protein of interest in transgenic tobacco plants, transgenic tobacco suspension cell cultures and transiently expressed in tobacco suspension cells. The flowchart of how to obtain a cell suspension in Fig. 3.1 can be easily adapted to other plant cell systems.

The gene expression system is stably transformed into *Nicotiana benthamiana* cells using *Agrobacterium*-mediated gene transformation by the recombinant *Agrobacterium tumefaciens* carrying appropriate binary vector containing the gene

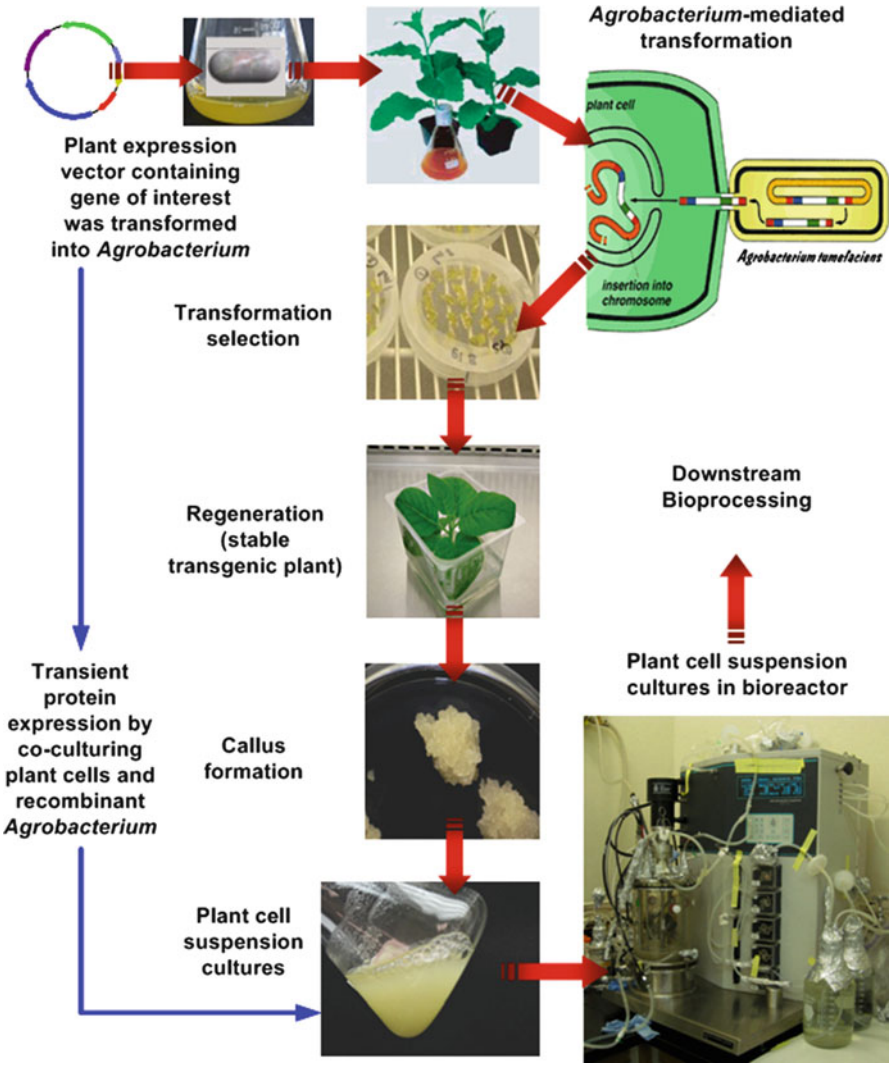


Fig. 3.1 A schematic presentation of the production of a recombinant protein of interest in transgenic plants, transgenic plant suspension cell cultures and transiently expressed in plant suspension cells

of interest. Newly expanded leaves from *N. benthamiana* plants are cut into 1 cm square sections soaked in an *Agrobacterium* solution adjusted to 0.1 OD_{600} for 10 min and incubated on co-cultivation medium consisting of Murashige and Skoog minimal organics (MSO) medium modified with 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BA), and 200 μM acetosyringone, pH 5.8, at 23–25°C in the dark for 2–3 days. Leaves are transferred to agar-solidified selection medium consisting of MSO medium modified with 30 g/L sucrose, 2 mg/L BA, 400 mg/L

carbenicillin, 250 mg/L cefotaxime, and 250 mg/L kanamycin and incubated at 23–25°C for 10 days. Plant tissues are subcultured until shoots formed. Shoots are harvested and transferred to agar-solidified rooting medium consisting of half strength MSO medium modified with 15 g/L sucrose, 2 mg/L BA, 1.3 g/L calcium gluconate, 400 mg/L carbenicillin, 250 mg/L cefotaxime, and 100 mg/L kanamycin. Leaves are removed from each rooted shoot with a portion of the petiole attached and placed on callus-generating medium consisting of MSO medium modified with 30 g/L sucrose, 0.4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin, 400 mg/L carbenicillin, 150 mg/L timentin, and 100 mg/L kanamycin for developing transgenic callus. Transgenic callus are subcultured every 3–4 weeks on agar-solidified KCMS medium consisting of 30 g/L sucrose, 4.3 g/L MS salt mixture, 0.1 g/L myo-inositol, 0.204 g/L KH_2PO_4, 0.5 mg/L nicotinic acid, 0.5 mg/L thiamine-HCl, 0.5 mg/L pyridoxine-HCl, 0.2 mg/L 2,4-D, 0.1 mg/L kinetin, and appropriate antibiotics as selection pressure, pH 5.8.

3.2.3.2 Transgenic Plant Cell Suspension Cultures and Media

Transgenic *N. benthamiana* cell lines are selected from transgenic callus cultures and screened by appropriate protein analysis methods such as ELISA and Western blots. There can often be a significant variation in expression levels between independent transformed lines due to the fact the that the expression cassette is inserted randomly at one or more locations in the nuclear genome, so screening of the independent transformation events, either at the whole plant or callus stage, is an important step. Transgenic plant cell suspension cultures established from callus cultures are subcultured weekly by transferring 20 mL of established suspension cells into 200 mL KCMS medium (pH 5.8), consisting of 30 g/L sucrose, 4.3 g/L MS salt mixture, 0.1 g/L myo-inositol, 0.204 g/L KH_2PO_4, 0.5 mg/L nicotinic acid, 0.5 mg/L thiamine-HCl, 0.5 mg/L pyridoxine-HCl, 0.2 mg/L 2,4-D, and 0.1 mg/L kinetin, and appropriate antibiotics as selection pressure, in a 1 L flask maintained on an orbital shaker at 140 rpm and 25°C. Scale-up of cell biomass expansion can be conducted in larger vessels such as stirred-tank bioreactor.

3.3 Optimization of Recombinant Protein Production in Plant Cell Cultures

Current limitations of plant cell-based expression systems for recombinant protein production are the low product yields and the effects of non-human glycosylation pattern of glycosylated plant-made recombinant proteins on the activity, immunogenicity and allergenicity for therapeutic applications. To achieve higher protein yields and desired quality, recombinant protein expressed in plant cells can be optimized from transcription to protein stability including the selections of promoters, enhancers, integration sites, codon usage/synthetic gene design, gene silencing suppressors, and product compartmentalization. Table 3.1 shows examples of

Table 3.1 Selected example of foreign protein productions by plant cell culture bioreactor systems (Huang and McDonald 2009)

Plant species	Culture type	Protein product	Promoter system	Localization	Bioreactor system	Operation conditions	Production level	Reference
Nicotiana benthamiana (tobacco)	Suspension cells	AAT	CaMV 35S	Secreted, extracellular	STR, pitched blade impeller	25°C, 50 rpm, 40% DO, pH 6.4, batch	<1 μg-FAAT/L (without pH control), 100 μg-FAAT/L (pH control)	Huang et al. (2009)
Nicotiana benthamiana (tobacco)	Suspension cells	AAT	Estradiol inducible XVE system	Secreted, extracellular	STR, pitched blade impeller	25°C, 50 rpm, 40% DO, pH 6.4, batch, 10 μM estradiol as inducer	<1 μg-FAAT/L (without pH control), 60 μg-FAAT/L (pH control)	Huang et al. (2009)
Nicotiana benthamiana (tobacco)	Suspension cells	AAT	Cucumber mosaic virus inducible viral vector (CMViva)	Secreted, extracellular	STR, pitched blade impeller	25°C, 50 rpm, 40% DO, pH 6.4, batch, 10 μM estradiol as inducer	25 μg-FAAT/L (without pH control), 100 μg-FAAT/L (pH control)	Huang et al. (2009)
Nicotiana benthamiana (tobacco)	Suspension cells	AAT	Cucumber mosaic virus inducible viral vector (CMViva)	Secreted, extracellular	STR, pitched blade impeller	25°C, 50–100 rpm, 40% DO, pH 6.4, SCC, 1 μM estradiol as inducer	600 μg-FAAT/L (pH control)	Huang et al. (2010)
O. sativa (rice)	Suspension cells	AAT	RAmy3D (inducible)	Secreted, extracellular	STR, pitched blade impeller	STR, 75 rpm, 27°C, 70% DO, 0.1–0.2 vvm, SCC (multiple growth and production phases)	40–110 mg/L, 3–12 mg/(L-day), 4–8 mg/(g-DCW-day)	Trexler et al. (2005)

(continued)

Table 3.1 (continued)

Plant species	Culture type	Protein product	Promoter system	Localization	Bioreactor system	Operation conditions	Production level	Reference
O. sativa (rice)	Suspension cells	AAT	RAmy3D (inducible)	Secreted, extracellular	Membrane bioreactor	25°C, 130 rpm, two stage culture (sugar starvation)	100–247 mg/L,4–10% of TSP	McDonald et al. (2005)
O. sativa (rice)	Suspension cells	hGM-CSF	RAmy3D (inducible)	Secreted, extracellular	Flask	110 rpm, 13 day, 27°C, batch	129 mg/L, 25% of TSP	Shin et al. (2003)
N. tabacum L. (tobacco)	Suspension cells	hGM-CSF	CaMV 35S	Secreted, extracellular	Flask	110 rpm, 12 day, 25°C, batch	105 μg/L	Hong et al. (2002)

AAT human alpha-1-antitrypsin; *FAAT* functional human alpha-1-antitrypsin; *DO* dissolved oxygen; *DCW* dried cell weight; *SCC* semicontinous culture; *D* dilution rate (1/day); *STR* stirred-tank bioreactor; *TSP* total soluble protein; *VVM* volume of gas per volume of culture per minute

recombinant protein production by stably transformed plant cell suspension culture in bioreactors, highlighting the importance of selection of host, expression vector and bioreactor operational strategy. Recent development to improve recombinant protein expression in plant cell cultures will be discussed.

3.3.1 Host Systems

Although tobacco, maize, rice and alfalfa are commonly utilized as hosts for recombinant proteins production, the emerging plant expression systems, like *Lemna minor*, *Physcomitrella patens*, *Chlamydomonas reinhardtii* or higher plant cell suspension cultures are offering new opportunities for molecular farming.

3.3.2 Genetic Transformation Methods

Several methods can be implemented for a recombinant protein expressed stably or transiently in plant cells including (1) stable transformation by incorporation of foreign DNA into nuclear, plastid or chloroplast genomes using *Agrobacterium*-mediated transformation, and by particle bombardment or electroporation of protoplasts (Sharma et al. 2005), and (2) transient expression by *Agrobacterium* agroinfiltration of plant tissue or infection with plant viral vectors (Pogue et al. 2010).

3.3.2.1 Stably Transgene Expression

Stable expression of a foreign gene in plant cell culture requires that the gene of interest is integrated into the nuclear or chloroplast genome of host cells and will be passed to subsequent generations. Transgenic plant-based molecular farming may be a better choice for long-term commercial scale production. The methods of plant transformation will depend on the plant host species and the procedure of stable transformation is well established and usually time consuming, which normally takes at least few months (3–9 months) for development and optimization of transformation, selection of a best transformed clone, and plant regeneration (Twyman et al. 2003).

However, genetic instability of the transgenic dedifferentiated cells has been shown to result in somaclonal variation (gene drift), a potential limiting factor in developing plant cell cultures for long term recombinant protein production (Offringa et al. 1990). Reduction in the product yield is often attributed to genetic instability, transgene loss, variation in growth rate, or other undesirable genetic or epigenic changes and has been observed for dedifferentiated plant cell cultures when maintained by liquid subculturing (Lambe et al. 1995; Vandermaas et al. 1994). Thus, cryopreservation of transgenic plant cell lines for plant cell suspension

culture has been discussed to allow for the long-term preservation and maintenance of a stable production system (Cho et al. 2007; Schmale et al. 2006). On the other hand, recombinant protein expression using transient expression in plant cell culture by either recombinant *Agrobacterium*-mediated transfection or plant viruses, common methods applied to whole plants and intact plant tissues (Komarova et al. 2010), is under development as an alternative for recombinant protein expression.

3.3.2.2 Transient Transgene Expression

Transient expression for rapid transgene gene expression has potential for large-scale recombinant protein production in whole plants or plant tissue. Transient expression by the agroinfiltration method (Kapila et al. 1997), in which recombinant *Agrobacterium tumefaciens* are infiltrated into plant tissue, or using plant viral vectors (Gleba et al. 2007) are commonly used. In agroinfiltration the T-DNA is transferred to the nucleus in a large number of plant cells resulting in the expression of milligram amounts of recombinant protein within a short time period (2–14 days depending on the protein of interest, the host and the expression system). Viral vectors have also attracted interest because viral infections are rapid and systemic, and infected cells yield large amounts of virus and viral gene products (Streatfield 2007). Since plant viruses do not integrate into the genome, there is no stable transformation and the transgene is not passed through the germ line.

Transient expression has given higher productivity than stably transformed plants mainly due to the relative timing of the onset of PTGS (post-transcriptional gene silencing) compared to transgene expression (Wroblewski et al. 2005) and position effect of transgene integration into the plant cell genome (Kumar and Fladung 2001). In transient expression, high levels of gene product may accumulate prior to the initiation of PTGS. Currently, only few examples of using transient expression for recombinant protein production in plant cell suspension cultures have been reported. Boivin et al. utilized transient co-expression of a transgene and the p19 viral suppressor by agroinfiltration to enhance the mouse IgG1 antibody production reaching 148 mg IgG1/kg-FW in *N. benthamiana* cell culture, implying the feasibility of using transient expression in a plant cell culture bioreactor system (Boivin et al. 2010).

3.3.3 Gene Expression Systems

The type and characteristics of the promoter system significantly impact the gene expression level by affecting the transcription rate of the target gene, and further determines the bioreactor optimal operation mode. Constitutive or inducible promoters are commonly utilized for gene expression in plant cell cultures. Table 3.2 compares the features of various promoters used in transgenic plants cells (Huang and McDonald 2009).

Table 3.2 Features of various promoters for expressing recombinant protein in plant cells (Huang and McDonald 2009)

Types	Examples	Features
Constitutive promoter	CaMV 35S, Maize ubiquitin	Commonly used in plant-based molecular farming
		Recombinant protein production is growth-associated
		Potential problem of PTGS
Inducible promoter (chemically)	Steroid-regulated	Easy for induction by simply adding inducer
		Low dose required in plant cell cultures
		Inducer-dependent toxic effects
	Ethanol-regulated	Inducer is simple with low toxicity
		Volatility may be a problem
	Tetracycline-regulated	Short half-life of antibiotics
		High dose required for induction, resulting in toxic effect to host cells
		Continuous addition required
Inducible promoter (metabolic)	Metabolite-regulated: rice alpha-amylase RAamy3D (sugar starvation)	Need to conduct media exchange
		Two stage cultures required
		Affect cellular metabolism of host cells
Inducible promoter (physical)	Temperature-regulated	Trigger heat shock response related proteins
		Influence cellular metabolism
		Additional energy required
	Light-regulated	Influence cell growth and cellular metabolism
		Additional energy required

Constitutive promoters, such as the CaMV 35S promoter, directly drive the expression of the target gene which is directly related to cell growth. The target protein expressed by a constitutive promoter is considered to be a growth-associated product and is continuously expressed until host cells reach the stationary phase, resulting in an additional metabolic burden and hence impacting the plant cellular physiology and growth rate due to the potential toxic properties of the product on the host cells or it may interfere with host cell metabolism.

Inducible gene expression systems controlled by the strength of specific external factors or compounds, such as light, temperature, metal ions, alcohols, steroids and herbicides, etc., have been developed to achieve high-level recombinant protein production (Murphy 2007; Padidam 2003; Zuo and Chua 2000), allowing the cell growth and protein production phases to be optimized independently. The inducible promoter systems are attractive particularly when product synthesis is deleterious to host cell growth. The expression of a foreign gene linked to an inducible promoter can be induced at a specific stage during the cell growth cycle, and there is less potential for PTGS found in transgenic plants driven by constitutive promoters (Vaucheret and Fagard 2001). Chemically inducible promoter systems induced by estradiol (Zuo et al. 2000), ethanol (Zhang and Mason 2006) and dexamethasone

(Samalova et al. 2005) have mainly been developed for recombinant protein production in plant systems. Selection of the highest expressing cell lines with an inducible promoter system is complicated by the fact that the optimal timing and level of the inducer is often not known *a priori* and must be determined experimentally.

Plant viral vectors, designed to increase the transgene copy number by the action of the viral replicase, are alternatives for foreign gene expression (Lico et al. 2008). Several genetically modified viral vectors, such as *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV) and *Potato virus* X (PVX), have been developed for recombinant protein production in plant molecular farming systems (Lico et al. 2008; Lindbo 2007; Wagner et al. 2004). However, plant viral vectors are prone to vector instability, leading to reduced replication of the viral vector due to methylation, PTGS, or loss of transgene (Angell and Baulcombe 1997; Atkinson et al. 1998).

Therefore, inducible plant viral vectors, which combine the features of inducible promoters and plant viral vectors (Gleba et al. 2007; Lico et al. 2008), are proposed as an alternative (Gleba et al. 2007). Our group has developed a *Cucumber mosaic virus* (CMV) inducible viral amplicon (CMViva) expression system encoding a viral replicase controlled by an estradiol-activated XVE promoter (Zuo et al. 2000), and hence the recombinant viral amplicons are only produced intracellularly under induction conditions. The CMViva system has been demonstrated to allow tightly regulated foreign gene expression and good production of functional recombinant human proteins in non-transgenic plant tissues using transient agroinfiltration and in transgenic tobacco cell suspension culture in bioreactor (Huang et al. 2009, 2010; Plesha et al. 2007, 2009; Sudarshana et al. 2006). Other examples of inducible plant viral vectors in plant cell cultures include the estradiol-inducible *Tomato Mosaic Virus* (ToMV) amplicon system expressing GFP in tobacco BY-2 cells (Dohi et al. 2006) and the ethanol-inducible *Bean Yellow Dwarf Virus* (BeYDV) amplicon expressing Norwalk virus capsid protein (NVCP) in tobacco NT1 cells (Zhang and Mason 2006).

3.3.4 Stability of Recombinant Proteins

Recombinant proteins expressed in plant cell cultures can be secreted to the extracellular culture medium or retained in an intracellular compartment such as ER, cytoplasm, or vacuole. Secreted products offer advantages of bioprocessing over intracellular retained products such as simpler downstream processing and flexibility in the bioreactor operational mode allowing continuous culture or multiple production cycles by reusing plant cells, leading to increased the overall productivity. However, secreted recombinant proteins are commonly degraded by proteolytic enzymes produced during plant cell cultivation or resulting from cell death/lysis (Doran 2006a) and/or may be unstable in the simple cell culture medium composition (James and Lee 2001; Tsoi and Doran 2002). Previous studies have proposed a variety of approaches to improve the stability of secreted recombinant proteins and

to prevent the recombinant protein loss from proteolytic degradation, including the supplementation of protease inhibitors or protein stabilization agents (such as gelatin, BSA and other low-value proteins), 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 produced from the cell cultures) (Doran 2006a, b). Other molecular-level approaches have also 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 (Komarnytsky et al. 2006), (2) suppression of protease gene expression using RNA interference (RNAi) (Kim et al. 2008), and (3) development of specific protease-deficient host cells (Schiermeyer et al. 2005).

Optimization of bioreactor operation is an alternative to enhance the stability of secreted recombinant proteins. Huang et al. (2009) proposed a bioreactor strategy involving pH control for improving functional recombinant human protein production in transgenic tobacco cell culture, resulting in enhanced recombinant protein stability and reduced protease activity in cell cultures, as an effective alternative to adding protease inhibitors or protein stabilizing agents in plant cell culture.

3.3.5 Post-translational Modification of Plant-Made Recombinant Proteins

Post-translational modification (PTM) of the plant-made protein is a critical quality attribute and in certain cases may limit the pharmaceutical and industrial applications of plant-made proteins. Although plants and mammalian cells share similar PTM mechanisms such as expressed proteins entering the secretory pathways where N-linked glycosylation occurs in the ER and Golgi apparatus, O-linked glycosylation occurs in the Golgi apparatus, and molecular chaperones in the ER help to fold the protein (Faye et al. 2005), minor differences in PTM have been observed. Plant cells tend to attach α-(1,3)-fucose and β-(1,2)-xylose in the glycan of the plant-made glycoprotein, which are absent in mammalian cells (Sethuraman and Stadheim 2006). In addition, plant-made glycoprotein lacks the terminal galactose and sialic acid residues, which have been observed on human glycoproteins (Gomord and Faye 2004). These differences in the glycan structures of plant-made glycoproteins may affect the expressed protein inherent stability, biological activity and immunogenicity, limiting their applications.

Strategies have been demonstrated to alleviate this issue by expressing "humanized" glycans in N-linked plant-made glycoproteins (Schahs et al. 2007), such as applying RNAi against to the appropriate fucose and xylose transferases in plant cells. Strasser et al. (2008) reported the generation of glyco-engineered *N. benthamiana* lines with downregulated endogenous xylosyltransferase and fucosyltransferase genes by using RNAi. In addition, double "knock-out" of the α-(1,3)-fucose and β-(1,2)-xylose transferases has also been investigated for the

production of human vascular endothelial growth factor (hVEGF) in moss cell culture (Koprivova et al. 2004). Another example is to delete the fucose residues in plant N-glycans by repression of the GDP-mannose 4,6-dehydratase gene using virus-induced gene silencing and RNAi (Gomord et al. 2010). Castilho et al. (2010) has introduced the entire biosynthetic pathway for sialyation and terminal galactose in *N. benthamiana* using transient expression for producing MAb with a human-like glycosylation pattern. Other strategies to control the glycosylated protein are to understand how variations of bioreactor process parameters affect protein glycosylation patterns in host cells (del Val et al. 2010).

3.4 Technological Progresses in Plant Cell Culture Bioreactor Systems

In this section, the technological progress in bioreactor systems including bioreactor selection, bioreactor operation considerations and optimization for recombinant protein production in plant cell cultures will be discussed.

3.4.1 Bioreactor Systems

Table 3.3 summarizes important features of various types of bioreactors for *in vitro* plant cell cultures. General criteria for choosing a suitable 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 by-product and metabolite removal from cells). The scalability of the bioreactor system for large scale production of a recombinant protein in plant cell culture also needs to be addressed.

3.4.1.1 Stirred-Tank Bioreactor

Stirred-tank bioreactors with suitable impellers can provide adequate volumetric mass transfer coefficients and a homogeneous environment enabling suspended plant cell growth and product production to be controlled. Although Rushton turbines (resulting in a radial flow pattern) can provide complete solids and gas dispersion, they also induce high turbulence around the impeller region and have higher specific power input and energy dissipation rate than other impellers with axial flow patterns (such as marine, paddle, and pitched-blade impellers), resulting in higher shear damage to suspended plant cells. The pitched-blade impeller with the upward-pumping mode provides similar capabilities, compared to Rushton turbines, for cell aggregate dispersion while reducing shear stress to plant cells when the power input was restricted by cell damage considerations (Doran 1999). However, the oxygen

mass transfer performance of an upward-pumping pitch-blade impeller was poor in highly cell density cultures (Kieran 2001), compared with that of the same impeller operated in the downward-pumping mode (Junker et al. 1998). Generally, impeller systems exhibiting axial flow patterns with low impeller tip speed (less than 2.5 m/s) are considered acceptable for plant cell cultures (Amanullah et al. 2004).

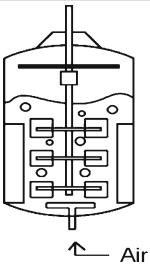
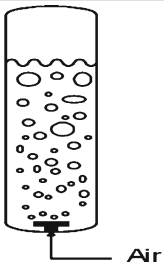
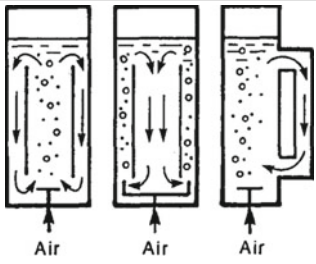
Approaches have been proposed to reduce cell damage from hydrodynamic shear stress by agitation and from gas bubble bursting by aeration including developing new impeller systems to provide more efficient mixing at lower impeller tip speeds (such as the curved-blade disk turbine, hydrofoil impeller, helical ribbon, centrifugal impellers and cell-lift) (Doran 1999) and designing new aeration systems (such as bubble-free aeration, gas basket, and cage-aeration) for shear-sensitive cell culture processes. In addition, the low-power-number impellers such as Intermig, Prochem Maxflow and Scaba designed for animal cell culture can be implemented for plant cell culture (Varley and Birch 1999). The bioreactor geometric specifications such as impeller diameter, spacing between impellers, impeller off-bottom clearance, the baffles and their width, the sparger type and position, the ratio of liquid height to tank diameter, and the number of impellers are also critical considerations for large-scale bioreactor performance in order to provide sufficient mixing and adequate mass transfer.

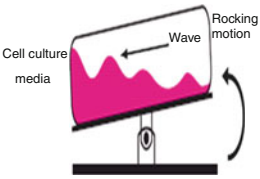
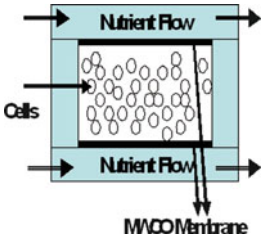
3.4.1.2 Pneumatic Bioreactor

The pneumatic bioreactor (such as a bubble column or air-lift), consisting of a cylindrical vessel in which air or a gas mixture is introduced through a sparger at the bottom of the vessel for aeration, mixing and fluid circulation, exhibits features of low capital and operational cost, and ease of scale up for large scale operation of plant cell cultures. In addition, the low shear stress in pneumatic bioreactors is desirable for shear-sensitive plant cells (Eibl and Eibl 2008). However, bubble column bioreactors are less applicable to highly viscous liquid and high cell density cultures due to the lower gas-liquid interfacial area resulting from bubble coalescence in the viscous liquids and lack of mechanical break-up of bubbles.

Air-lift or modified air-lift bioreactors containing a draft tube (internal or external loop) exhibit the following features: (1) preventing bubble coalescence by directing them in one direction; (2) enhancing oxygen mass transfer by increasing the number of bubbles or gas-liquid interfacial area for enhancing mass transfer; (3) distributing shear stress more evenly; and (4) promoting the cyclical movement of fluid resulting in shorter mixing times (Huang et al. 2001, Wang et al. 2002). However, the inadequate oxygen mass transfer and poor fluid mixing in a high cell density culture, 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 (Tanaka 2000).

Table 3.3 Comparisons of bioreactor systems for foreign protein production by plant cell cultures

Bioreactor	Stirred-tank	Bubble column	Air-lift bioreactor
Features			
$k_L a$ (OTR)	High	Low	Medium
Cell damage by agitation	High	Low	Low
Cell damage by aeration	Medium	High	High
Mixing time	Short	Long	Medium
Operation	Medium	Simple	Simple
Flexibility	High	High	High
CIP/SIP	Yes	Yes	Yes
Scale size	Commercial	Commercial	Commercial
Scale-up	Medium	Easy	Easy
Power consumption	High	Low	Low
Culture type	Suspension; microcarrier	Suspension; microcarrier	Suspension; microcarrier
Cell density	Low/medium	Medium	Medium
Productivity	Medium	Medium	Medium
Monitoring and control	Direct, easy, multiple	Direct, easy, multiple	Direct, easy, multiple
Operation cost	High	Medium	Medium
Equipment cost	High	Medium	Medium
Ease of GMP compliance	Yes	Yes	Yes

Single use technology	Wave bioreactor	Membrane bioreactor
		
Low	Medium	Low
Medium	Low	Low
Medium	Low	Low
Medium	Medium	Medium
Medium	Medium	Medium
Medium	Medium	Low
No	No	No
Pilot (2,000 L)	Pilot (1,000 L)	Pilot
Medium	Complex	Complex
Medium	Medium	Low
Suspension; microcarrier	Suspension; microcarrier	Immobilized
Low/medium	Medium	High
Medium	Medium	High
Direct, easy, limited	Direct, medium, limited	Indirect, complex, limited
Low	Low	Low
Low	Low	Low
Yes	Yes	Difficult

3.4.1.3 Disposable Bioreactor (Single-Use Bioreactor)

Disposable bioreactors, in which the cultivation vessel is made of a single-use plastic bag, provide attractive features for the biomanufacturing production of recombinant proteins in terms of time- and cost-savings for clean-in-place, sterilization-in-place, facility design and set-up, validation, capital investment on stainless steel vessels, elimination of cross-contamination and reduction of turnover time between each run, and shorter development time and increased throughput (Eibl et al. 2009a, b; Hacker et al. 2009). Currently disposable bioreactors have been successfully implemented into preclinical, clinical, and production-scale biomanufacturing facilities (Eibl et al. 2010). Although disposable bioreactors with standard stirred-tank configurations up to 2,000 L are available from companies such as HyClone and Xcellerex, they are limited in volume and mostly used for seed expansion and inoculation of the large conventional bioreactors. The disposable shaken bioreactor is another type of disposable bioreactor, which consists of a cylindrical vessel mounted on a circular moving shaker platform and contains a disposable, sterile plastic bag with appropriate connection tubes for seeding, feeding, gas supply and harvesting of these cultures (Micheletti et al. 2006). Protalix utilizes a disposable, bubble column-type bioreactor, which consists of a sterilizable polyethylene bag filled with plant cells and medium, for the prGCD production in transgenic carrot cell culture (Shaaltiel et al. 2008).

3.4.1.4 Wave Bioreactor

Wave bioreactors, another type of disposable bioreactor, possessing the advantages of low-cost and providing a low shear environment, utilize a non-gas permeable sterile bag comprised of plastic film. The mixing, mass and heat transfer in the wave bioreactor are regulated by rocking rate, rocking angle and medium filling volume. The bags can be filled with cell suspension up to 50–60% of their total capacity (up to 1,000 L) (Eibl et al. 2009a). Oxygen is supplied from the air or gas mixture continuously through headspace aeration. While the wave bioreactor is rocking, the liquid 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 suspended cells. Additional advantages include time and cost savings, reduced foaming, easy operation and low risk of contamination. The wave bioreactor can be operated in different culture modes, including batch, fed-batch and perfusion when combined with different cell-retention devices. On-line measurements of pH, DO and other sensing technology (Read et al. 2009) make the wave bioreactor and other disposable bioreactors highly attractive for plant cell cultures (Eibl et al. 2009a). Investigations have demonstrated the application of the wave bioreactor for cultivating tobacco, grape and apple suspension cells up to 100 L working volume (Terrier et al. 2007). Eibl et al. 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 (Eibl and Eibl 2006).

3.4.1.5 Membrane Bioreactor

Membrane bioreactors are designed to retain host cells and also possibly recombinant protein product in a cell compartment by utilizing specialized membranes with a specific molecular weight cut-off (MWCO) for *in situ* aeration, nutrient supply, and product separation (Qi et al. 2003). The culture medium flow is circulated in the membrane bioreactor for bringing oxygen and nutrients to the cell and removing the waste metabolites. Membranes can be packed into different geometries including plate-and-sheet, tubular, spiral-wound, and hollow-fiber modules. The hollow fiber membrane bioreactor is the most commonly used geometry.

The main features of using membrane bioreactors in plant cell cultures are high cell density and high protein volumetric productivity, resulting from the use of membranes that retain the secreted foreign protein in the cell compartment, concentrating the product before harvest. Another feature is that the shear stress-induced cell damage found in stirred-tank bioreactors can be minimized in a membrane bioreactor because the cells are retained in a relatively quiescent region in which cells are protected from mechanical damage and are not in direct contact with gas bubbles. McDonald et al. (McDonald et al. 2005) 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 up to 250 mg/L (equivalent to 4–10% of the extracellular total soluble protein). However, large scale process in membrane bioreactors may cause operation problems resulting in poor cell viability, poor process stability, product heterogeneity, membrane fouling, and diffusion gradients (heat and mass transfer). Therefore the membrane bioreactor is mainly implemented for small scale processes and is difficult to scale up for large scale applications, although it may be useful for smaller scale applications such as patient-specific therapeutics.

3.4.2 Bioreactor Operation Consideration

Plant cell cultures exhibiting features of biological, morphological properties and cellular physiology characteristics, which are distinctive from bacterial and mammalian cells, need to be considered for their scalability in recombinant protein production. Important bioreactor operation considerations for plant cell cultures include hydrodynamics, mass and heat transfer, mixing, cell growth, viability and oxygen demand, cell aggregation, rheological properties and shear sensitivity of plant cell cultures, etc.

3.4.2.1 Plant Cell Growth and Oxygen Demand

Plant cells show a longer doubling time (20–100 h) than that of bacterial (30 min to 1 h), yeast (2–3 h) and mammalian cells (24–48 h). Different species show varied

cell growth kinetics in bioreactors. A typical OUR (oxygen update rate) value for plant cell cultures is about 2–10 mmol-O_2/(L-h), compared with 10–90 mmol-O_2/(L-h) for microbial cells and 0.05–10 mmol-O_2/(L-h) or 0.02–0.1 × 10–9 mmol/(cell-h) for mammalian cells (depending on the cell density and cell line type). The specific OUR is reported as 0.8 mmol-O_2/(g-DCW-h) for transgenic rice cell cultures expressing human AAT (Trexler et al. 2002) and 0.3–0.5 mmol-O_2/(g-DCW-h) for transgenic NT-1 cells expressing recombinant GUS. Inadequate oxygen mass transfer has been demonstrated to inhibit transgenic tobacco cell growth and reduce recombinant antibody heavy chain production (Sharp and Doran 2001b). Gao and Lee (1992) found that an increase in the oxygen supply enhanced tobacco cell growth rate, biomass concentration, and GUS production yield in shake flask, stirred-tank and air-lift bioreactors.

To meet the OUR of 2–10 mmol-O_2/(L-h) for plant cell cultures, a typical volumetric oxygen mass transfer coefficient (kLa) required in a bioreactor operation is between 10 and 50 h^{-1} (Curtis and Tuerk 2006), which is lower than that for microbial fermentation (100–1,000 h^{-1}) and slightly higher than that for mammalian cell culture (0.25–10 h^{-1}). The critical dissolved oxygen concentration for plant cell cultures in a bioreactor is reported as 1.3–1.6 g/m^3, corresponding to 20% of air saturation (Doran 1993). The optimal settings of kLa and dissolved oxygen concentration in bioreactor for plant cells expressing recombinant protein need to be investigated individually.

3.4.2.2 Aggregation and Rheological Properties of Plant Cell Cultures

Plant cell aggregate formation and aggregate size distribution are critical for plant cell culture bioreactor operation and are dependent on plant species, method of inoculum preparation, cell growth stage, medium composition, bioreactor types and culture conditions. Formation of plant cell aggregates promotes cellular organization and differentiation resulting in enhancing secondary metabolite production, and also impacts mass transfer, leading to oxygen, nutrient or chemical inducer inhomogeneities inside large cell aggregates (Kieran 2001). Therefore, the inner cells of the aggregates may become oxygen and nutrient deficient, resulting in adverse effects on cell growth and foreign protein production. Although moderate cell aggregation (200–500 μm) is advantageous in some cases 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, exacerbates mass transfer limitations and makes cell aggregates more susceptible to shear stress, resulting in cell damage, attributed to aggregate surface attrition (Kieran et al. 2000) and aggregate shattering (Namdev and Dunlop 1995).

Rheological properties of plant cells *in vitro* 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 (Cosgrove 1997). Curtis (Curtis and Emery 1993) studied

the rheological properties of 10 different plant cells in flasks and found that elongated plant cell morphology in *N. tabacum* batch culture exhibited a power-law type fluid rheological property (with a power law index of 0.6), resulting in higher apparent viscosity, compared to spherical cells. Curtis (Curtis and Emery 1993) also observed that tobacco cell culture displayed Newtonian rheological properties and did not elongate when grown in semicontinuous culture (cells remained nearly spherical in shape), confirming the dependence of rheology on cell morphology. Elongated plant cell morphology may lead to higher packed cell volume (PCV) at a given dried cell weight (DCW), attributed to a more loose cellular network under packed conditions (Su and Arias 2003).

Kato et al. (1978) and Curtis (Curtis and Emery 1993) both found that culture spent media was not responsible for the overall broth viscosity and the viscous and non-Newtonian fluid character of the culture was mainly due to the plant cell morphology (elongated and filamentous cells) and high biomass concentration (with DCW over 10 g/L and PCV over 50–60%). Kato et al. (1978) found 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.

3.4.2.3 Shear Sensitivity of Plant Cell Cultures

Two main shear stresses leading to cell damage in plant cell culture bioreactor operation are hydrodynamic shear force induced by agitation and air bubble bursting caused by gas sparging. 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 (Dunlop et al. 1994). 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 (Wagner and Vogelmann 1977), inducing the cellular response changes including cell viability, cell growth rate, membrane integrity, release of intracellular components (proteins or metabolites), metabolism (OUR, mitochondrial activity, ATP concentration, cell wall composition, increase of calcium ions in cytoplasm), cell morphology and aggregation sizes (Kieran et al. 1997, 2000), influenced by the intensity and the exposure duration of the cells to the shear force.

For a stirred-tank bioreactor, shear stress generated by the impeller system reduces the average aggregate size of *Catharanthus roseus* cell culture (80–100 μm in the shake flask versus 64–80 μm in the stirred-tank bioreactor) and has adverse effects on plant cell growth and viability (Tanaka et al. 1988), partially due to the fact that plant cells are subject to the higher shear stress region of the impeller and thus more

shear-induced damage on cells is generated (Doran 1999). For shear-sensitive plant cell cultures, therefore, reducing the shear stress intensity by decreasing the agitation speed of the impeller is a general solution, while maintaining adequate mixing, oxygen and heat transfer rates in a high apparent viscosity plant cell culture broth. At high biomass concentration, low agitation rates can also enhance the clumping of cells into cell aggregates of varying sizes. Thus, a 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/m^2 has been reported (Kieran et al. 1997).

3.4.3 Bioreactor Process Optimization

The optimization of bioreactor operation needs to consider the characteristics of plant cell expression system such as cell growth, viability and oxygen demand, cell aggregation, rheological properties and shear sensitivity of plant cell cultures, foaming and wall growth, interactions between host cell, gene expression system and product formation including the type of gene expression system, expressed product location, impact of expressed products on host cells, etc.

For the growth-associated recombinant protein production (secreted or intracellular product) driven by a constitutive promoter, target protein productivity can be enhanced by reaching high cell density culture 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 (Suehara et al. 1996). However, the accumulation of inhibitory metabolites in fed-batch cultures might limit recombinant protein productivity. Therefore, perfusion culture with a cell retention device can be an alternative to obtain high cell density culture and continuously withdraw cultured medium (De Dobbeleer et al. 2006; Lucumi and Posten 2006; Su and Arias 2003). The cell growth rate in a batch or fed-batch plant cell culture is usually reduced when the PCV reaches about 60–70%, resulting in a reduction of cellular metabolic activity (Maccarthy et al. 1980). Therefore, semi-continuous culture or perfusion culture with a bleed stream has been demonstrated to be more appropriate for high cell density culture compared with fed-batch and perfusion cultures (De Dobbeleer et al. 2006).

For recombinant protein production driven by an inducible promoter, two-stage cultures are typically implemented to allow the cell growth and protein production phases to be independently optimized. The bioreactor operation and conditions for the induction phase (protein production phase) are highly dependent on the type of inducible promoter used (Huang and McDonald 2009). The sugar-starvation Ramy3D promoter system (metabolically regulated) has been investigated to express human proteins including human AAT (Huang et al. 2001) and human granulocyte-macrophage colony stimulating factor (hGM-CSF) (Shin et al. 2003) in transgenic rice cell cultures. In these studies, a media exchange to a sugar-free medium or nutrient medium containing an alternative carbon source (Terashima et al. 2001) for

inducing foreign protein production was applied at an appropriate 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. Therefore, a cyclical semi-continuous process, which alternates between growth and production phases, has been developed to reuse the transgenic rice cells for long-term operation (Trexler et al. 2005). For a chemically inducible system, the timing of induction and concentration and manner of application of inducer (single, multiple or continuous induction) applied to plant cell cultures are important for optimizing inducible plant cell culture bioreactor operation. This will need to be investigated based on the nature of the inducer (inducer stability and toxicity to host cells) and plant species (cell growth rate and aggregates) for enhancing foreign protein 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 beneficial for inducible production of the recombinant protein and the secreted recombinant protein can be continuously harvested from the cell culture broth. Huang et al. showed that OUR is an important parameter to determine the optimal timing of induction (TOI) (Huang et al. 2010). 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 (Huang et al. 2010). We developed the semicontinuous culture production of human AAT using a chemically inducible plant viral vector in transgenic tobacco cell culture, resulting in fivefold increase in volumetric productivity of biologically functional AAT compared with batch operation (Huang et al. 2010).

Additionally, the productivity of secreted recombinant proteins, which could be 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 (Benchabane et al. 2008; Doran 2006a, b), (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) (James et al. 2002; Sharp and Doran 2001a), and (3) immobilization of plant cells, in which cells are immobilized into a suitable microcarrier or support matrix in a bioreactor (Gilleta et al. 2000; Osuna et al. 2008), to facilitate recovery of secreted protein from the culture broth.

3.4.4 Scale Up Considerations

The development of the large-scale bioreactor operation of plant cell suspension culture has been well established for the production of plant secondary metabolites such as paclitaxel (taxol), ginseng and shikonin (Hellwig et al. 2004). Phyton Biotech (www.phytonbiotech.com) commercially produces paclitaxel compound using plant cell suspension cultures up to 75,000 L and has been a long-term

supplier of this small molecule API (active pharmaceutical ingredient) of Bristol-Myers Squibb's Taxol® oncology product. Therefore, the bioreactor technologies available for large scale plant secondary metabolites production could be implemented for the large scale production of recombinant proteins in transgenic plant cell suspension cultures. Currently, the most challenging problem of the scale-up of bioreactor-based plant cell culture is to providing a low-shear environment while maintaining adequate mixing and oxygen transfer in high cell density culture and/or in long-term perfusion culture operation. Although some investigations have tried to meet market demands by increasing bioreactor capacity, the optimization of cell culture productivity in bioreactors appears as a better strategy.

3.5 Current Status of Commercialization

Although molecular farming using bioreactor-based plant cell suspension cultures provides an alternative biomanufacturing platform for large scale production of recombinant proteins for pharmaceutical and industrial applications, only few available commercial examples have been demonstrated.

In February 2006, USDA approved the first transgenic tobacco cell culture-produced recombinant glycoprotein by Dow AgroSciences (www.dowagro.com), as a veterinary vaccine based on the HN antigen derived from immunoprotective particles of Newcastle Disease Virus (NDV) for preventing avian NDV disease (Travis 2008). Other successful examples from Dow AgroSciences for the production of immunogenic proteins include the HA antigen 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 (Mihaliak et al. 2007). The recombinant immunoprotective proteins are expressed and accumulated in the stationary phase of plant cell growth in cytoplasmic, cell wall or membrane structure, with the product titer up to 4–30 mg/L (Mihaliak et al. 2007; Miller et al. 2006).

The most recent case in the plant cell culture made pharmaceuticals is the transgenic carrot suspension cell culture in bioreactors developed by Protalix Biotherapeutics for recombinant glucocerebrosidase (GCD) production dedicated for patients with the genetic disorder Gaucher disease (Shaaltiel et al. 2007). Protalix Biotherapeutics (http://www.protalix.com/) in Israel and Pfizer in the US (http://www.pfizer.com/) have announced a collaboration to market the recombinant glucocerebrosidase enzyme produced by transgenic carrot cell cultures as a therapeutic protein drug, currently in phase III clinical trial, for the treatment of Gaucher's disease in EU and USA (Ratner 2010). This represents an exciting milestone for recognizing plant cell culture-based biomanufacturing as a bio-equivalent and economical alternative to mammalian production of human biopharmaceuticals, further suggesting the possibility of biosimilar products for existing protein drugs. Currently patients are treated with either Ceredase® by Genzyme purified from

human placental tissue or Cerezyme® by Genzyme produced in recombinant CHO cell cultures (Ratner 2010). However, the recombinant GCD by CHO cell cultures requires an additional *in vitro* enzymatic reaction to expose the terminal mannose residues of its N-glycan chains to facilitate uptake of the GCD into macrophages, making it one of the most expensive therapeutic proteins to date with an annual treatment cost of nearly US $200,000 per patient (Kaiser 2008). The plant-made recombinant GCD (prGCD) expressed in transgenic carrot cell culture by Protalix is fused to the N-terminal signal peptide from *Arabidopsis thaliana* basic endochitinase and to a C-terminal vacuole targeting sequence from tobacco chitinase A. The prGCD expressed by transgenic carrot cells is retained in ER (endoplasmic reticulum) for glycosylation and then targeted to the vacuole. Therefore, the N-glycan structures of the prGCD are trimmed to expose mannose residues, leading to the correct mannose glycosylation pattern. As a result, the *in vitro* trimming of the glycans for *in vitro* protein modification is eliminated during downstream processing, resulting in significant cost reduction (Shaaltiel et al. 2007). Currently, the prGCD by Protalix is undergoing a Phase III clinical trial to evaluate its safety and efficacy in Gaucher patients.

3.6 Future Prospects

Though cheaper, safer, easier to manipulate and more rapid than most established molecular farming using transgenic plants, plant cell suspension culture is still not the best production platform the plant system can offer, as the overall product yield and usability is often limited by the loss of recombinant protein during the late stationary phase due to increased proteolytic activity (Corrado and Karali 2009). Although the adoption of advanced cell culture technology has been implemented during the past years, the system is still limited to a small number of well-characterized plant cell lines (such as tobacco, rice, carrot, or *Arabidopsis*), which are amenable to develop suspension cell cultures and need improvement before they can be adopted commercially.

Therefore, future directions for enabling the molecular farming using bioreactor-based plant cell suspension cultures as a recombinant protein production biomanufacturing platform include: (1) selection of plant hosts with lower endogenous protease activity to the target protein, (2) development of algorithms for synthetic gene design for optimal expression in plant hosts, (3) generation and selection of the most productive cell lines by automatic high throughput system and/or development of site-specific integration strategies, (4) medium formulation design and optimization, (5) enhancing recombinant protein stability and preventing proteolytic degradation, (6) selection of bioreactor systems according to the interactions between host cells, product formation and bioreactor design, (7) optimization of bioreactor operation strategy, (8) incorporation of gene silencing suppressors, (9) development of large scale transient expression in plant cell culture, (10) adaptation of disposable bioreactor technology, and (11) engineering humanized plant-made glycosylated proteins.

3.7 Conclusions

The promising cases described above, including Protalix's transgenic carrot suspension culture platform for prGCD production, indicate the opportunities for molecular farming using plant cell culture bioreactor system for large scale biomanufacturing of specialty proteins, orphan drugs or personalized medicine, rare genetic diseases, and biosimilars or even biobetter therapeutics, to lower the cost of goods while maintaining or improving the plant-made protein quality. Plant cell culture bioreactor systems exhibit the advantage of plant-made therapeutics in a similar method capable of meeting the EMEA and FDA regulatory requirements that have been set for the past 20 years for microbial and mammalian cells-made proteins. Strategies for the selection of plant species, cell culture types, gene expression systems, bioreactor systems and operation modes, and product of interest for application need to be carefully investigated for making the plant cell bioreactor processes a practical and economical platform for foreign protein production in next phase of development and commercial application.

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