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Reza S. Gharelo¹
Ebrahim D. Oliaei¹
Ali Bandehagh¹
Ehsaneh Khodadadi¹
Pouya M. Noparvar^{1,2}

Production of therapeutic proteins through plant tissue and cell culture

Authors' addresses:

¹Department of Plant Breeding and Biotechnology, University of Tabriz, 29 Bahman Blvd., Tabriz 5166616471, East Azarbaijan, Iran.

²Young Researchers and Elite Club, Tabriz Branch, Islamic Azad University, Tabriz 5166616471, Iran.

Correspondence:

Reza Shokri Gharelo
Department of Plant Breeding and Biotechnology, University of Tabriz, 29 Bahman Blvd., Tabriz 5166616471, East Azarbaijan, Iran
Tel: + (98 411) 3392031
Fax: + (98 411) 3356003
e-mail: shokrireza67@gmail.com

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ABSTRACT

Nowadays, pharmaceutical recombinant protein is increasingly used in treatment of many diseases such as hepatitis, anemia, diabetes and cancer. Different protein expression systems have been used for the expression of recombinant proteins in which each of them face obstacles that make utilizing them as comprehensive expression system in order to express wide variety of proteins difficult.

Plant cell as a eukaryotic expression system have many advantages compared to other hosts. They are very "safe" and significantly decrease concerns about the contamination of recombinant proteins with human pathogens. In addition to this, plants as eukaryotic expression system perform proper post-translational modification, in case of eukaryotic proteins, and appropriate folding resulting in right function in biological environments. Therefore, the production of pharmaceutical protein through plant cells can be absolutely promising approach. In this review, the production of pharmaceutical protein in plant cells, advantages and disadvantages, offered methods and techniques for developing recombinant protein yields, and affective factors on the whole process of pharmaceutical protein expression in the molecular level will be reviewed.

Key words: bioreactor, plant suspension culture, recombinant protein

Introduction

Proteins have various roles in cells and carry out wide range of biological reactions from taking part in structural components to involving in defense mechanisms of the organism against invaders. Any defection of these proteins can lead to various harmful diseases. Using recombinant proteins in order to treat these type of diseases compared to chemically synthesized drugs have many advantages such as high specificity, correct function, non-interpretation with other biological reactions, and non-induction of immunological responses (Leader *et al.*, 2008). Recombinant protein production for clinical purpose made revolution in modern medicine. Recombinant proteins have been used for treating various diseases such as hepatitis, anemia, diabetes, and cancers (Xu *et al.*, 2011). Medicinal and industrial application of recombinant protein is rising along with rising

demand of world market leading to emerge of various protein expression systems and other related high-tech systems to date (Karen *et al.*, 2012). Annually trading pharmaceutical proteins overtake more than 90 billion. It has been predicted that it would catch up with 125 billion by the 2015 (Xu *et al.*, 2011).

E. coli has commonly been used as a prokaryotic expression system for the production of recombinant proteins. Average amount of obtained protein from *E. coli* is 39% of total soluble proteins (TSP). *Saccharomyces cerevisiae* and *Pichia pastoris* are mostly used as eukaryotic hosts for recombinant protein production. Their average amount of production is about 15% of total soluble protein (Scotti *et al.*, 2012). Nowadays, commercial production of recombinant proteins, on the other hand, is conducted by mammalian and bacterial cell cultures, but proteins expression in these organic systems have many problems, e.g. costly production, and potentiality

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infected product by pathogens. Consequently, using plants as a promising expression platforms to produce recombinant proteins look necessary (Xu et al., 2011).

Plant biotechnology made second metabolites and recombinant proteins available to produce in plants as cheap-and-powerful bioreactors through genetically modified plants (Sharma & Sharma, 2009). Using transgenic plants have been turned to practical strategy in these years. Although some obstacles constrain utilizing plant systems, there are still advantages to introduce these green creatures as economic hosts for alien gens. Problems, for instance longevity of transgenic plant growth and development, high diversity in the amount and quality of produced recombinant proteins, risk of infection by herbicides, pesticides, plant diseases, and chemical fertilizers along with depending on weather, soil, and season conditions, are association with whole plant expression systems (Hellwig et al., 2004). From another view it could claimed that problems could be solved by novel biotech approaches along retaining advantages of whole plant expression systems as well. In this case, plant cell/tissue

cultures are appropriate choice for expression of recombinant protein because of having plant platforms advantages with overcoming their drawback.

Nowadays, therapeutic proteins and industrial enzyme are produced by tissue culture, which mentioned to some of them in Table 1 (Karen et al., 2012). β -glucocerebrosidase (GCD) (EC 3.2.1.45) is plant cell-based product that is used orally for treatment of Gaucher disease. Carrot cells are genetically engineered to express the GCD enzyme and used as a delivery vehicle to reach it into the human bloodstream as well. PR-112, which is a plant cell-expressed form of GCD, acquired its verification from U.S. Food and Drug Administration (FDA) in 2009 (Tekoah et al., 2015). Plant cell and tissue cultures in comparison with other systems have advantages, including low cost of production, performing post-translational modification, independence from weather, soil, and other environmental conditions, low possibility of being infected by herbicides, pesticides, diseases, and chemically fertilizers (Hellwig et al., 2004). In addition, simple extraction and purification of recombinant

Table 1. *Produced recombinant proteins through plant cells and plant tissue culture.*

Recombinant protein	Host	Protein yield	Reference
Human serum albumin	<i>Nicotiana tabacum</i>	0.25 mg/l	Dekker et al., 1990
Human erythropoietin	<i>N. tabacum</i> cv. BY-2	1 pg/dry weight	Matsumoto et al., 1995
Bryodin 1	<i>N. tabacum</i> cv. NT-1	30 mg/l	Francisco et al., 1997
Antibody svFv fragment	<i>Oryza sativa</i> cv. Bengal	3.8 μ g/callus dry weight	Vaquero et al., 1999
Human α 1 antitrypsin	<i>O. sativa</i>	200 mg/l	Huang et al., 2001
Human interferon α 2b	<i>N. tabacum</i> cv. BY-2	28 mg/l	Xu et al., 2007
Human growth hormone	<i>O. sativa</i> cv. Donjin	57 mg/l	Kim et al., 2008
Cytotoxic T cells surface antigen	<i>O. sativa</i>	76.5 mg/l	Park et al., 2010
Human lysosome	<i>O. sativa</i> cv. Taipie	3-4% TSP	Huang et al., 2002
Human alkaline phosphatase	<i>N. tabacum</i> cv. NT-1	27 mg/l	Becerra-Arteaga et al., 2006
Human collagen α 1 chain	<i>Hordeum vulgare</i>	2-9 mg/l	Wahlström et al., 2008

Table 2. *Plant cell and tissue culture technique in comparison to other common systems to recombinant protein expression.*

Host	Overall cost	Producti on time	Scale-up capacity	Product quality	Contamination risk	Purification cost
Plant cells	medium	medium	high	high	very low	medium
Whole plants	low	very long	very high	high	low	high
Mammalian cells	high	high	medium	very high	very high	high
Bacteria	low	low	high	low	medium	high

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proteins, especially once secreted to culture medium is recognized as promising characteristic (Doran, 2000). High quality-products and ability to controlling system are as important as low-cost production (Fischer *et al.*, 2004). Table 2 indicates plant cell and tissue culture technique in comparison with other common expression systems for recombinant proteins.

Plant cell and tissue culture

Most studies have been focused on the production of recombinant proteins by non-differentiated tissue such as callus and suspension cultures (Doran, 2000), but differentiated culture are considered too. Cell suspension culture (non-differentiated) and hairy root culture (differentiated) have high potential for production of recombinant proteins (Weathers *et al.*, 2010).

Differentiated tissue culture of plants has advantages including high growth rate, high expression level, and genetic stability. Mouse interleukin-12 protein has been produced using hairy root culture of *Nicotiana tabacum* cv. BY-2 by 1.5 total soluble proteins (Liu *et al.*, 2009). Nevertheless, suspension cell culture is preferred to hairy root culture because of compatibility with the standards and capacity for conducting in the bioreactors. Cell suspension cultures of tobacco, alfalfa, rice, tomato, and soybean have been used for production of recombinant proteins (Terashima *et al.*, 1999; Kwon *et al.*, 2003). Tobacco NT-1 and BY-2 cell lines mostly are used as expression systems to produce foreign proteins since they possess high growth rate, high expression level, efficient secretion of foreign proteins to outside of cells, and also are easily infected by *Agrobacterium* (Lee *et al.*, 2007; Ozawa & Takaiwa, 2010; Tremblay *et al.*, 2010). Tobacco BY-2 cell lines are obtained from calluses of BY-2, which is a cultivar of tobacco, and propagated in modified LB medium (Nagata & Kumagai, 1999). These lines are commonly used in studying of plant cells cycle and biology (Sorrell *et al.*, 1999). Fragile calluses are a suitable type of callus to use in cell suspension cultures. Transgenic plants that express protein of interest can be used for callus resource at suspension culture. Moreover, callus can be obtained from non-transgenic plants and be transformed using co-culture technique with *Agrobacterium tumefaciens* (Hellwig *et al.*, 2004). The major issue in the suspension culture method is genetic instability during long-time use (Weathers *et al.*, 2010).

Therapeutic proteins which are produced in plant suspension cell cultures

There are many reports about recombinant protein production in plant cells. Antibodies, vaccines, hormones, and cytokines are important recombinant proteins, which have been produced by using plant cells culture (Table 2). After expression of human serum albumin in tobacco cell culture by Sijmons *et al.* (1990), many attempts performed for the production of other human proteins in plant cell cultures. Various pharmaceutical proteins have been produced using plant cell culture in which hormones have essential importance (Table 3).

Antibodies

Antibodies are proteins that are produced in response to foreign factors (i.e. antigens) by B-lymphocytes in vertebrates. The antibodies have high variety and selectivity. They perform important roles in identification and elimination of foreign substances in acquired immune system (Abbas *et al.*, 2012). Increasing concerns about probable infection of mammalian and bacterial-based expression systems lead to plant cell/tissue culture-based systems as safe expression systems attract scientific attention. The first monoclonal antibody production was reported in the cell culture of tobacco leaf (Hiatt *et al.*, 1989). Subsequently, immunoglobulin A and G were produced in cell suspension culture of *Nicotiana* and *Arabidopsis* (De Wilde *et al.*, 1998; de Jaeger *et al.*, 2000). Monoclonal antibody against surface antigen of hepatitis B (Yano *et al.*, 2004) and HIV are such produced antibodies using the suspension culture of plant cell through the tobacco suspension cell culture (Holland *et al.*, 2010). Table 4 represents other types of plant cell-based produced antibodies.

Vaccines

Vaccines are antigenic substances that providing passive immunity in vertebrate to protect them from special detrimental antigens (Abbas *et al.*, 2012). Vaccine production using plant systems have two main advantages: first, merely selected immunologic epitope presents in plant, and second, it is possible to express therapeutic proteins in plants and then to use them as a delivery vehicle (Tiwari *et al.*, 2009). Produced vaccines in plant systems maintain advantages such as controllability, sterility, production reproducibility, and compatibility with GMP manufacturing requirements (Tiwari *et al.*, 2009). Intimin production in suspension cell culture of NT-1 lines can be mentioned as an example. In mouse,

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Table 3. Some important recombinant proteins which have been produced, recently.

Recombinant proteins	Host cells	Protein yield	Reference
Human alpha-l-iduronidase	Tobacco BY-2 cells	10 mg/l	Fu et al., 2009
Human HIV antibody	Tobacco BY-2 cells	10 mg/l	Holland et al., 2010
Human interleukin 12	Rice	31 mg/l	Shin et al., 2010
Human growth hormone	Tobacco BY-2 cells	35 mg/l	Xu et al., 2010
Human epidermal growth hormone	Tobacco	2 µg/dry weight	Parsons et al., 2010
β-Galactosidase	Tobacco	not reported	Lee et al., 2007
Tanomatin	Tobacco	2.63 mg/l	Pham et al., 2012

Table 4. Some recombinant antibodies produced using plant cell/tissue culture.

Recombinant protein	Host	Reference
Human anti-rabies virus mAb	Tobacco	Girard et al., 2006
ScFv antibody	Tobacco	Firek et al., 1993
LO/BM2, a therapeutic IgG	Tobacco	De Muynck et al., 2009
MAK33, IgG	Potato	De Wildo et al., 2002
CO17-1A, IgG2a	Tobacco	Ko et al., 2005
cPIPP, IgG1	Tobacco	Sriraman et al., 2004
H10, IgG1λ	Tobacco	Villani et al., 2009

Table 5. Some recombinant vaccines produced using plant cell-based platforms.

Recombinant protein	Host	Reference
Hepatitis B surface antigen	Potato	Richter et al., 2000
VP1 epitope of foot and mouth disease virus	Potato	Carrillo et al., 2001
B subunit of cholera toxin of <i>E. coli</i>	Tomato	Jani et al., 2002
B subunit of the heat liable toxin of <i>E. coli</i>	Maize	Streatfield et al., 2002
B subunit of the heat liable toxin of <i>E. coli</i>	Lettuce	Martinez-Gonzalez et al., 2011
Rheumatoid arthritis type II collagen	Rice	Hashizume et al., 2008
Capsid precursor polypeptide of FMDV	Rice	Wang et al., 2012

immune system induction and activation occur when with these cell lines are inoculated mice (Judge et al., 2004). Some of the plant cell-expression vaccines are represented in Table 5.

Cytokines, growth hormones, and growth factors

The cytokines are wide group of secretory proteins that are produced by various types of immune cells. They play an important role as signaling molecules to regulate innate and acquired immune system. Important types of cytokines are interleukins (Abbas et al., 2012). The hIL-12 (human

interleukin-12) in rice suspension cell culture (Shin et al., 2010) and hIL-10 (human interleukin-10) expressed in tobacco BY-2 cells have been reported (Kaldis et al., 2013). In addition, human growth hormone production in rice cells (57 mg/l) and tobacco BY-2 cells (35 mg/l) have been reported (Yeoun et al., 2008; Xu et al., 2010). Erythropoietin expression using cell culture of *Medicago truncatula*, *Arabidopsis thaliana* and tobacco cell lines have been reported as well (Pires et al., 2012). Human growth hormones expression in hairy root culture of plants such as *Nicotiana benthamiana* also has been performed (Skarjinskaia et al.,

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2008). Hairy roots can be used for long-time-hosts due to genetic stability of root tissue for production of recombinant proteins (Georgiev *et al.*, 2013).

Therapeutic enzymes

Several enzymes with therapeutic and diagnostic applications have been produced using plant cell and tissue culture. Human tissue transglutaminase (EC 2.3.2.13) (htTG) is an example of these enzymes which is prescribed to coeliac disease treatment. The htTG is expressed artificially in BY-2 cell (Sorrentino *et al.*, 2005). Acetylcholinesterase (EC 3.1.1.7) is another example that is produced using hairy root culture of *N. bethamiana* (Woods *et al.*, 2008). In addition, human lysozyme (EC 3.2.1.17) with antimicrobial effects has been produced in both rice whole plant and grains (Huang *et al.*, 2002).

Strategies for high yield production of recombinant proteins using plant cell culture

Despite advantages of using plant cell and tissue culture platforms, there are several limitations for this type of the expression systems, including formation of cell aggregation, cells tendency to adhere to the bioreactors' walls, diversity, genetic instability, and gene silencing (Hellwig *et al.*, 2004). The majority of these issues can be solved using appropriate bioreactors, selection of suitable cell lines, and optimization of culture medium. The main problem of using plant cell and tissue cultures is the low amount of produced proteins in comparison to the whole plant platform (Hellwig *et al.*, 2004), which restricts therapeutic protein products to be commercialized (Xu *et al.*, 2011). Many efforts have been conducted to increase the yield of recombinant protein expression in plant cell/tissue-based expression systems. Using special manipulation strategies, optimization culture/growth conditions of plant cells as well as exploiting

specialized bioreactors for plant cell cultures are considered as promising methods to achieve high yield of recombinant protein production, and some of them will be discussed in the coming section.

Enhancing gene transcriptions

Increasing gene transcription is an efficient approach in order to gain high expression level of recombinant proteins. Transcription process is the first stage in which gene expression level is regulated. Many factors have influence on this process, such as promoters, transcription initiation factors, and enhancers. Exploiting the potential of powerful promoters could be an efficient strategy to increase gene expression rate. Totally, the promoters, which are used in plant systems, are categorized into two groups: constitutive promoters, and inducible promoters (Egelkroust *et al.*, 2012).

Constitutive promoters drive continually genes expression and acts independently from stimulus and environmental agents (Table 6). This type of promoters is used in different tissues. 35S promoter from cauliflower mosaic virus is well known example and is recurrently used for expressing recombinant proteins in plants. It has been reported that duplication of the 35S promoter sequence improves the gene transcription rate up to 10-fold (Kay *et al.*, 1987). Constitutive promoters have been used in dicotyledons including canola, tobacco, and *Arabidopsis* with up to 7% total soluble protein yield (Odell *et al.*, 1985), and in monocotyledons such as wheat and maize with up to 1.13% total soluble protein yield (Ransom *et al.*, 2007). Ubiquitin and (ocs) 3mas promoters are another constitutive promoters that have been used in plant cell culture. The ubiquitin promoter is obtained from maize, *Arabidopsis*, potato, sunflower, tobacco, and rice (Sharma & Sharma, 2009). It has been used for expression of scFv antibody and insulin like growth factors-1 in rice cell culture (Torres *et al.*, 1999;

Table 6. Some constitutive promoters and inducible promoters had been used to recombinant proteins production (Xu *et al.*, 2011).

Promoters	Therapeutic protein	Host cell	Protein yield
CaMV35S	Human interferon α 2b	<i>N. tabacum</i> cv. BY-2	28 mg/l
	Human growth hormone	<i>N. tabacum</i> cv. BY-2	35 mg/l
	Byodin 1	<i>N. tabacum</i> cv. NT-1	30 mg/l
	Human alkaline phosphatase	<i>N. tabacum</i> cv. NT-1	27 mg/l
(ocs)3mas	Hepatitis B surface antigen	<i>Glycine max</i> cs82v William	22 mg/l
RAmy3D	Human growth hormone	<i>O. sativa</i> L. Donjin	57 mg/l
	Human α 1 antitrypsin	<i>O. sativa</i>	200 mg/l
SWPA2	Human lactoferrin	<i>Acanthopanax senicosus</i>	3.6% TSP
	Human lactoferrin	<i>N. tabacum</i> cv. BY-2	4.3% TSP

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Panahi *et al.*, 2004). Another type of the constitutive promoters is the (ocs) 3mas promoter which consists of the octopine synthase promoter and the mannopine synthase from *Agrobacterium*. It has been used for hepatitis B surface antigen (HBsAg) expression in soybean cell culture (Smith *et al.*, 2002).

Inducible promoters are affected by stimulus such as tetracycline, alcohol, steroids, salt, sucrose, and environmental factors such as temperature, light, oxidative stress, and wounding (Table 6). Usage of the inducible promoters is more common than the constitutive promoters because of ability to control the gene expression, especially if the secreted and accumulations of recombinant proteins are toxic for the cells. The inducers for promoter induction must possess some important characteristics including high specificity, rapid response of promoters, non-toxicity for plant cells, and easy scale-up. The RAmy 3D promoter, from α -amylase (EC 3.2.1.1) encoding gene in rice embryo, is activated in sucrose starvation conditions. This promoter has been used for expressing some therapeutic proteins in rice cell suspension cultures, including rAAT, hGM-CSF, Bryodin-1, lysozyme, and serum albumin (Lee *et al.*, 2003; Yeoun *et al.*, 2008; Sharma & Sharma, 2009; Karen *et al.*, 2012). In addition, the stress-inducible peroxidase promoter has been used for expression of human lactoferrin in tobacco BY-2 cells (Choi *et al.*, 2003). It was observed that this promoter is up to 30-fold more active than the 35S promoter (Lee & Kim, 2006). XVE and pOp/LhG4 promoters have also been used in plant cell cultures which are induced by estradiol and dexamethasone, respectively (Zuo *et al.*, 2000; Samalova *et al.*, 2005).

mRNA processing and stability

mRNA processing includes capping, splicing, and adding poly-A tail, has influence on expression level of the recombinant proteins. Downstream sequences of gene act as signal for addition of the poly-A tail. Added poly-A tail give stability for mRNA and consequently increase the gene expression level by providing more opportunity for ribosome to be bound mRNA in cell cytosol (Desai *et al.*, 2010). Therefore, the untranslated region (UTR) can also be manipulated in order to increase the expression level of recombinant protein. The presence of some sequences in mRNA can reduce the half-life of mRNA in cell's cytosol. It has been reported that the elimination of AU-rich sequence from 3'-UTR prevents mRNA degradation (Lin *et al.*, 2009).

Reducing post-translational degradation of recombinant proteins

Protein degradation after translation can lead to significantly decrease in total protein content. Increasing number of mRNA transcriptions alone cannot guarantee high recombinant protein accumulation in cells, since there is possibility that successfully expressed proteins undergo degradation processes. Proteolytic enzymes play important roles in accumulation of proteins produced in cells and culture medium (Doran, 2006). Sharp & Doran (2001) reported degradation of IgG1 antibody in tobacco hairy root and cell suspension culture.

Protein degradation can be because of the protein presence in specific organelles such as vacuole. Vacuole contains wide variety of proteolytic enzymes that are activated under acidic conditions. It has been reported that vacuole may be a location for proteolysis in potato and *A. thaliana* (Outchkourov *et al.*, 2003; Yang *et al.*, 2005). Apoplast and cell walls also produce proteolytic elements (Stevens *et al.*, 2000). Protein degradation occurred in endoplasmic reticulum is less than in other organelles, hence, fusion of signal peptides (KDEL or HDEL) within recombinant proteins to drive them into endoplasmic reticulum can result in less degradation of recombinant proteins (Doran, 2006; Sharma & Sharma, 2009; Desai *et al.*, 2010). In addition, chaperons and stabilizers in endoplasmic reticulum confer more stability and correct folding for secreted protein. Using this method can increase 10 to 100-fold more recombinant protein expression. For instance, it has been reported that human epithelial growth factor (EGF) fused to KDEL sequence, represent 104-fold more expression level, compared with those proteins without this signal peptide (Doran, 2006; Streatfield, 2007).

Co-expression of recombinant proteins with protease inhibitors, creating mutation in protease encoding genes, and elimination of active sites of the proteases using genetic engineering techniques are other strategies in order to reduce protein degradation. Expressing protease inhibitors reduced protease activity in lettuce (Van der Vyver *et al.*, 2003). Co-expressing recombinant proteins with their subunits or cofactors is another method to prevent proteolytic activities. In plant cells, those proteins that do not bind to their own subunits or cofactors undergo degradation process (Gruis *et al.*, 2002). It has been reported that co-expressing ricin A and B chains and antibody with their own antigens resulting in those proteins stabilization and less degradation (Frigerio *et al.*, 1998).

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Tagging recombinant proteins for secreting into medium not only reduce the cost of purification and proteins recovery, but also phenolic and oxidative substances are eliminated and cell lysis stage is circumvented. However, protein secretion into medium exposes them to proteolytic activities (Hellwig *et al.*, 2004). For overcoming this problem, adding inhibitors in medium is one of the strategies for protecting secreted proteins from degradation. It has been reported that adding 1000 milligram bactericide into tobacco cell culture medium result in more than 2.5-fold production of hGM-CSF (Lee *et al.*, 2003). Protease inhibitors and ethylenediaminetetraacetate (EDTA) supplements in *N. tubacum* cv. BY-2 cell culture medium has boosted α -plasminogen production (Benchabane *et al.*, 2008).

Using stabilizer substances in medium is another strategy to increase recombinant proteins which are secreted into the culture medium. Gelatin, bovine serum albumin, mannitol, polyethylene glycol, pluronic F-68, and other polymers have been used as stabilizers in order to protect recombinant proteins from denaturation (Lin *et al.*, 2009). Protease inhibitors are also used for this purpose, but they have short half-life and their scale-up is costly (Ikemura, 1985). Using RNAi technology to block protease gene transcripts, utilizing mutated cell for protease genes, early recovering of produced protein from medium, and plant cells immobilization are another alternative strategies that have been used for declining recombinant protein degradation to date (Doran, 2006; Xu *et al.*, 2011).

Optimization codon usage

The ratio of codons for a given amino acid differs from one organism to another. The level of tRNA available for that amino acid correlates to the amino acids ratio (Liu & Xue, 2005). Codons are used in the plants differ from animals and even between monocotyledons and dicotyledons (Jabeen *et al.*, 2010). Therefore, using codons compatible with host codon usage and removing rare codons can have more influences on recombinant protein expression level (Geyer *et al.*, 2007). Codon usage optimization can be performed using analysis of the available sequences (Jabeen *et al.*, 2010). There is various software for this purpose. Some of them are mentioned here: DNAWorker, Gene Designe, GeneDesign, and OPTIMIZER (Egelkroust *et al.*, 2012). The optimization of codon usage for human acetylcholinesterase (EC 3.1.1.7) in tobacco has increased its expression up to 5-10-fold (Wolin & Walter, 1988).

The rare codons form secondary structures in encoding

regions of the gene by which the movement of ribosome on mRNA sequence is slowed down (Chen & Inouye, 1990). The translation process even can be halted because of rare codons presence (Kang *et al.*, 2004). Hence, elimination of rare codons can significantly elevate the transgenic gene expression in plants (Mason *et al.*, 1998).

Optimization of plant cell culture medium

Standard plant cell/tissue culture media such as MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), B5 (Gamborg *et al.*, 1968), and LS (Linsmaier & Skoog, 1965), are commonly used. Modifying composition of these standard culture media can increase the expression level of recombinant proteins (Vasilev *et al.*, 2013). It has been reported that nitrogen source optimization, increase 10 to 20-fold antibody expression for HIV in tobacco BY-2 cell culture medium (Holland *et al.*, 2010). In the case of IgG1 production has also been reported that culture medium modification resulting in increased 5-fold yield (Doran, 2000). Vasilev *et al.* (2013) reported that medium salts including KNO_3 , NH_4NO_3 , CaCl_2 and other compounds like 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) significantly affect accumulation of M12 human antibody and increase 20 to 30-fold its accumulation in comparison to the standard MS medium.

Using compatible bioreactors for plant cell cultures

Bioreactor is a vessel that is used for providing an optimized environment for biological processes and is used to produce and engineer biologic products. Generally, all bioreactors are composed of culture tank, airing systems, stirred systems, and equipment for entrance and exit of the liquid culture medium. In plant biotechnology, bioreactors are used for plant cell/tissue cultures. Bioreactors should to provide optimal environment for the plant cell growth for high yield production of recombinant proteins. Generally, bioreactor systems are categorized in two groups: reusable bioreactors and disposable bioreactors.

The reusable bioreactors are made of stainless steel and can be used recurrently for recombinant protein production. Commonly, the reusable bioreactors are utilized at industrial-scale with 1000 to 25000 liter working volume (Doran, 1999).

Stirred-tank bioreactors, pneumatic bioreactors, and fixed-bed bioreactors are types of reusable bioreactors. Stirred-tank bioreactors are equipped with horizontal and vertical impellers that provide high mass transfer coefficient and uniform suspension of the cell culture medium (Eibl &

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Eibls, 2008). The pneumatic bioreactors consist of cylindrical tank in which compressed gas and air entrance provides suitable aeration (Meuwly *et al.*, 2007). Fixed-bed bioreactors consist of appropriate constant bed such as microcarriers in which cells, tissues, and organs of plants are fixed. Circulating liquid nutrients across beds provide nutrient and oxygen for cultured cells/tissue or organ. The major disadvantage of fixed-bed bioreactor is its low mass transfer coefficient and heat transfer (Eibl *et al.*, 2010).

Disposable bioreactors consist of single-use plastic bag or container and provide some advantages over reusable bioreactors including easy handling, no need for pre-sterilization, simple facility design, fast set-up, and time/cost saving (Eibl & Eibl, 2006). Wave bioreactor, membrane bioreactor, and rotating drum bioreactor are examples of disposable bioreactors, which have been used for production of recombinant proteins by culturing plant cells (McDonald *et al.*, 2005; Parekh *et al.*, 2008).

Selection of the type of proper bioreactor relies on plant cells characteristics and recombinant protein features. There are important criteria that should be considered for bioreactor selection and design, including low growth rate of plant cells, low capability for oxygen absorption, large size, and morphological complexity, shear sensitivity, and tendency to form cell aggregation and adhesion to the bioreactor walls (Ritala *et al.*, 2008).

Appropriate culture medium mixing is an essential factor to uniform distribution of nutrients and preventing metabolites to be accumulated. Controlling pH, temperature, and material concentration depend on medium mixing process. Different bioreactors possess different equipment for medium mixing. Uniform distribution of nutrients is a major problem in large-scale productions. Inappropriate mixing of medium can result in nutrients heterogeneous spreading and low mass transfer. High stirring intensity can solve this problem, but not for those cells, which are shear sensitive. Aeration and oxygen availability for plant cells are also important factors especially when cultured plant cells have high density. Hence, oxygen transfer coefficient must be regarded to bioreactors selection and design. In the other word, the design should be commensurate with plant cell properties. Among bioreactors, wave bioreactor is commonly used for plant suspension cell cultures. Using wave bioreactor in barley suspension cell culture in order to produce human α -1 collagen has resulted in 5 μ g/l human α -1 collagen protein (Wilken & Nikolov, 2012).

Recovery and purification of produced proteins

Recovery and purification of target recombinant protein is an important stage especially for the therapeutic proteins. This stage can account 80% of total production costs and even more in the case of therapeutic proteins that need to meet 99% purity (Hellwig *et al.*, 2004). Therefore, efforts to develop and improve recovery and purification methods can significantly decline the total cost of recombinant proteins production. Recombinant protein concentration and medium components are effective factors on recovery and purification processes. When the recombinant protein is trapped inside the cell, the first stage would be the cell disruption. Techniques available for plant cell disruption include sonication, pressure homogenization, wet milling, and enzymatic lysis. While all methods work efficiently in plant cell disruption, wet milling and sonication are difficult to scale-up and the enzymatic lysis is high costly in large scale. In the case of the secreted recombinant proteins into the medium cell disruption is circumvented (Saxena *et al.*, 2009). Subsequently, centrifugation, at small-scale, or dead end/cross flow filtration, at large scale, is carried out. The purification steps are carried out in order to increase recombinant protein purification. Several chromatography methods, including ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, and size exclusion chromatography are used for clarification of the recombinant protein. Furthermore, adding a polypeptide tail to the N or C terminus of proteins can be recruited using genetic engineering techniques for efficient separation of proteins. The tail characteristics are used to separate the recombinant proteins of interest from other proteins or substances (Ford *et al.*, 1991).

Conclusion

The rising production of therapeutic recombinant protein is speeded up day-by-day due to the increasing of world market demand. Among the different expression systems, plant cells are considered as a most promising platform for safe production of recombinant proteins. However, the suspension cell/tissue culture of plant faces many obstacles to reach profitable commercialization. In the last decade, many studies have been conducted in order to develop plant cell/tissue suspension cultures in order to optimize the production of recombinant protein yield. Development of cell and tissue culture techniques, genetic engineering, recovery, purification, and designing compatible bioreactors with plant

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cell/tissue cultures make hopeful prospective to produce the recombinant proteins with adequate yield for large-scale production.

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