

Review

Plant Biofarming: Novel Insights for Peptide Expression in Heterologous Systems

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ABSTRACT:

Peptide expression methods have been widely studied and developed from many different biological sources. The cultivation of prokaryotic and eukaryotic cells has proven to be efficient for the expression of foreign peptides in several heterologous systems, including bacteria, insects, yeasts, and mammals. Earlier reports brought up new insights for the improvement of expressed products to not only increase the production rate of desired peptides but also reproduce desirable post-translational modifications and even to reduce the risk of allergenicity when those products are aimed for human use. The development of bioreactor systems provided the optimization of cell growth conditions to scale up the amounts of expressed peptides. On the other hand, different cell systems and mutants provided a plethora of possible peptide modifications. Hence, in this report, we describe the many organisms and systems used for the large scale production of several macromolecules with relevance in health and agriculture. We also bring into discussion plant biofarming in the moss *Physcomitrella patens* and its recent adaptations, as a cost-effective and efficient approach in the production of more complex heterologous proteins, given the fact that its glycosylation pattern can be engineered to avoid allergenicity to humans (common to plant-derived glycoproteins). © 2012 Wiley Periodicals, Inc. *Biopolymers (Pept Sci)* 98: 416–427, 2012.

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INTRODUCTION

Since the early 80's, researchers have been taking advantage of the development of recombinant DNA technology and studies in the control of gene expression to direct the production of desired peptides in heterologous systems. Reports on the understanding of viral promoters and the possibility to clone these promoters to specifically enhance the expression of a single heterologous peptide have opened up the field of expression of heterologous peptides.^{1–6} Since then, different kinds of organisms ranging from bacterial cultures to higher plant species and mammalian cell cultures have been extensively studied to understand their specific genome properties and molecular machinery to better suit the expression profile needed for each peptide. Because of the vast knowledge of its genetics and molecular biology, *E. coli* has been widely used as a cellular host for peptide expression since the early beginnings of peptide expression and large scale production of heterologous peptides.^{3,5–10} Nevertheless, the application of this system for certain eukaryotic peptides is problematic, once that this microorganism lacks complex post-translational tools, as well as a multifaceted way for peptide transport and secretion from the cell. Therefore, to obtain peptides that require more specific folding features, post-translational modifications and differential intra and extracellular targeting, were also under intense study. These include opti-

mization of the already successful bacterial system and the exploitation of other organisms and cell lines. One classic example is the adaptation of one organism to perform certain peptide modifications, which can be observed on the development of hybrid pro- and eukaryotic peptide processing signals. Hence, the process used to induce a bacteria to cleave pre-proinsulin to proinsulin¹¹ opened the possibility for the expression of eukaryotic post-translationally modified peptides in bacteria. The use of different organisms, such as yeast^{12–17} and mammalian cell lines, came as alternative ways to produce recombinant eukaryotic peptides in large-scale, enabling the desired expression levels of macromolecules with the correct fold and complex modifications, including high content of disulfide bonds, specific glycosylation and/or phosphorylation patterns.

In the past three decades, peptide production in heterologous systems has been object of intense studies that yielded enormous advances in the field. However, these improvements have produced little in terms of the host choices for peptide expression, which remain basically the same of those used in the mid-80's. The great change since then is the amount of peptide produced, which has enormously increased due to processes that are significantly more productive.^{18,19} Lately,¹⁸ modifications in media content and the application of different cell/tissue feeding strategies allowed constitutive productions of recombinant peptides for longer periods and with healthier cells, providing the maintenance of productivity capacity.

Indeed, recent studies have evidenced the use of bioreactors as very cost effective procedure to produce high amounts of recombinant peptides using several different types of cells and organisms,^{18,20–25} especially for biopharmaceutical products,^{21–23,25–27} which are of great interest for industry and public health. Initially, seed plants were the major organisms used in bioreactors, but, nowadays, mosses and microalgae are occupying the first position, not only being selected for peptide expression^{21,22,28,29} but also for bioenergy production.^{30–33}

Therefore, in this report we review the most widely used systems for production of recombinant peptides, their main uses, advantages and disadvantages. Moreover, we will address the specificity each cell system developed for certain peptide groups, as well the use of bioreactor as a novel tool for large-scale peptide production.

CELL CULTURE SYSTEMS FOR PEPTIDE EXPRESSION

Bacterial Cells

Prokaryotic expression systems are usually the first method of choice for targeting foreign genes through genetic trans-

formation and directing the expression of the desired peptide because of its well established and straightforward protocols. Among these microorganisms, *Escherichia coli* is the most used bacterium for targeting gene overexpression, provided that its genetics is very well studied, which allows high expression levels coupled with abundant peptide productivity at relatively low costs. Furthermore, it takes advantage of a versatile variability of vectors systems and host strains^{34–36} available in the market. Usually, eukaryotic systems are used only when the peptide of interest requires specific modifications that cannot be performed by prokaryotic systems.³⁴ However, although high levels of eukaryotic peptides can be achieved using *E. coli*, it is not a common characteristic of this system. In general, the production of a foreign peptide in prokaryotic systems yields around 10% of the total cellular peptide.³⁴ The production of antibodies using prokaryotic systems has proved to be as efficient as using eukaryotic cells. In 1999, it was shown that a scFv fragment of a monoclonal antibody against tomato spotted wilt tospovirus could be highly expressed in *E. coli* cells.³⁷ Although the main purpose of the antibody production was targeted in plant systems, the results demonstrated that active antibody fragments can be easily obtained through bacterial cells.

Prokaryotic systems have some other limitations, such as the need for adaptation of its codon usage and low stability of the expressed product. Some gene products can also cause toxicity to the bacteria, inhibiting cell growth and high-level of peptide expression. Furthermore, the expressed product forms inclusion bodies, adding further solubilization procedures to recover the peptide of interest³⁴ (Table I). Nevertheless, most of these problems can be circumvented by avoiding the addition of rare codons in *E. coli*, choosing appropriate expression vectors and by redesigning or modifying target genes.^{35,36,38} A novel alternative in prokaryotic systems has been the introduction of the fusion peptides strategy, where suitable ligands for the peptide can facilitate solubilization and increase expression levels.³⁴

Another disadvantage of choosing bacterial systems for protein expression is the lack of post-translational modifications, protein maturation and stability. Nevertheless, to circumvent these problems, many strategies have been applied to optimize bacterial expression processes.³⁹ In this way, it was demonstrated that some important biopharmaceutical products can be successfully produced into bacterial cells and present same activity without the need of glycosylation. This was observed for interleukin-2 (INL-2) and β -interferon (INF- β).⁴⁰ In other cases, protein mutations allowed the expression of active and soluble products into *E. coli* cells, such as the recombinant mutant of human erythropoietin (rhEPO), where the residues sites of N-linked glycosylation

Table I Advantages and Disadvantages of Protein Heterologous Systems

Heterologous System	Advantages	Disadvantages
Bacterial	Well-studied system High diversity of vectors Easy control of cell expression Rapid growth of cell mass (4–24 h) High protein production (up to 500 mg/L culture) Relatively cheap	Limited capacity to form disulfide bonds No other post-translational modifications Formation of inclusion bodies Do not secrete recombinant proteins Limited to small inserts
Yeast	Easy to manipulate, when compared with other eukaryotic cell systems Allow post-translational modifications (disulfide bonds, N- and O-glycosylations, protein maturation) Secretion of recombinant proteins High protein expression (up to 1 g/L) in bioreactors	Codon usage Nondesired post-translational modifications Low levels of secretion for endogenous proteins
Mammalian cells	Efficiency on the production of recombinant human proteins Post-translational modifications Best for glycosylation and protein folding High production quality	High cost when compared with bacterial and yeast systems Cells required long periods to growth (2–3 weeks) Difficult for manipulation Low level of cell mass production
Very low scale-up capacity		
Insect cells	Allow the expression of more than two genes at the same time High level of expressed proteins Ability of using bigger inserts into the vectors Easy to purify Expression can be easily monitored High production quality	High cost Difficulty on cell manipulation Long production time scale

were substituted by other amino acids, maintaining its structure and activity.⁴¹

Although prokaryotic systems are relatively easy to use and their methods are fast improving to provide the production of a widespread variety of peptides, it is still not recommended for expression of membrane-associated peptides, mammalian peptide kinases, phosphodiesterases, and any peptide that requires complex post-translational modifications for its proper activity.

Yeast Cells

The introduction of yeast cell cultures for the expression of recombinant peptides offered significant advantages over bacteria and mammalian systems. It enabled the production of soluble and correctly folded foreign peptides that need post-translational modification, mainly glycosylations, which were not obtained in bacterial cells (Table II). Furthermore, it allows the insertion of linearized foreign DNA with high efficiency via homologous recombination procedures, generating stable cell lines. Yeast cells are easier to culture and genetically manipulate when compared with mammalian cells,

providing the same pattern of cell growth density.^{42,43} *Pichia pastoris* has been the most widely used yeast for expression of recombinant peptides. Indeed, *P. pastoris* present significant advantages over bacterial systems, especially for peptides with multiple disulphide bonds or requiring glycosylation, phosphorylation, when there is no oligomer formation for the correct folding or no aminoterminal methionine.⁴⁴ This system yields high amounts of peptides in the soluble phase, if correct yeast strains and expression vector are chosen. It is widely used for the study of peptide structures through X-ray diffraction crystallographic analyses, protein–protein interaction and on the assembly of peptide complexes, among others.⁴⁴ Moreover, it allows the insertion of multiple copies of the target gene in the same expression vector, resulting in high amounts of soluble recombinant peptide, properly folded and with most of the post-translational modifications. These combined features also reduce the costs of the system (Table I).

In addition, *P. pastoris* system has been used as a biocontrol agent expressing foreigner peptides that act against pathogenic fungi. For instance, recent studies showed that

Table II Post-translational Modifications Done by Yeast, Insect, and Mammalian Cells

Cells	Post-translational Modifications							Proteolytic Cleavage	Vitamin Dependent Modification
	Phosphorylation	Acetylation	Methylation	Sulfation	Prenylation	Glycosylation			
Yeast	X	X		X		X			
Insect	X	X			X	X	X		
Mammalian	X	X	X	X	X	X	X		X

P. pastoris is an efficient system for the production of a corn defensin PDC1 that was able to inhibit the activity of *Fusarium graminearum*.⁴² Furthermore, a defensin called AX2, isolated from sugar beet leaves, was successfully expressed and correctly folded in *P. pastoris* expression system. As this peptide contains a high number of disulfide bonds, its correct folding was a concern. *P. pastoris* could not only express and secrete AX2 properly, as it correctly folded the peptide, which was further confirmed by laser desorption mass spectrometry.⁴⁵ Another recombinant defensin, isolated from pea and called Pds1, was expressed in *P. pastoris*, demonstrating its use as a biocontrol agent against the apple decay caused by *Penicillium expansum*.⁴³

Insect Cells

The first description of insect cell culture dates back to 1917 and consisted on the cultivation of cells using simple salt solutions for the study of gametogenesis in tissue cultures.^{46,47} Later, improvements on culture medium based on insect tissue biochemistry provided the establishment of various cell lines from lepidopteran, diptera, orthopteran, and coleopteran insect orders, and also on the propagation of viruses in cultured cells.^{48–50} Nowadays, insect cell lines are used for a wide variety of purposes, such as viral propagation and optimization for the development of viral pesticides, on the studies of virus-cell interactions and in the production of recombinant peptides and vaccines^{48,49,51–54} (Table I).

Hence, the use of *Spodoptera frugiperda* cell lines (*Sf9*) has been widely used for the production of recombinant peptides coded by the adeno-associated virus genes (rAAV).⁵⁵ Peptide expression is achieved by taking advantage of baculovirus expression vectors (BEV) and has already proven to be cost-effective with successful results already available in the market. For instance, up to date, the pharmaceutical compound CervarixTM (GlaxoSmithKline) is the only product established in insect cells approved in Europe for human use.^{56,57} However, the company Amsterdam Molecular Therapeutics b.v. is developing studies using *Sf9* lines producing rAAVs for type I hyperlipoproteinemia, which is caused by a lipoprotein lipase deficiency.⁵⁷ Moreover, middle- and large-

scale production of rAAVs can be achieved by using several different bioreactors. The use of WaveTM system has achieved the best results for rAAV production in insect cells.⁵⁸ This type of bioreactor does not demand a strong previous knowledge on bioprocessing and it is very simple to manipulate when dealing with single-use sterile bags. The protocol for insect cells growth in bioreactors is already well recorded and has been utilized for culture between 1L and 25L.^{58,59} An alternative method for insect cell culture is the use of stirred tank bioreactors (STBR). Although this system has a higher cost and needs a more complex infrastructure to work with, it allows an accurate control of several parameters during the process (temperature, agitation speed, pH, and dielectric spectroscopy) that maintains a uniform production of the recombinant peptide. Furthermore, it permits the production of hundreds to thousands of liters of the desired culture.⁵⁷ Nevertheless, for rAAV production using STBR, a maximum of 40L production has been documented so far.^{58,60,61}

Recently, insect cell lines were successfully used to express a viral surface protein hemagglutinin to develop biopharmaceutical products against the influenza virus. *Spodoptera frugiperda* *Sf9* insect cells were infected with Autographa californica nucleopolyhedrovirus (AcMNPV). This methodology avoided the risk of virus contamination, maintaining the effectiveness for vaccine production.⁶²

Mammalian Cells

Most of the recombinant peptides expressed for therapeutical use are obtained by yeast or microbial cell culture systems, such as the case of commercial human insulin. Hence, most common therapeutic peptides expressed in heterologous systems are blood factors, growth factors, vaccines, hormones, anticoagulants, cytokines, and monoclonal antibodies. However, these systems are not able to perform all of the modifications required for proper peptide structure and function. Therefore, they cannot carry out extensive, complex and/or specific post-translational modifications that mimics those performed in human cells, such as the complex glycosylation patterns.⁶³ N-linked glycosylation is uncommon in bacterial

cells, whereas yeast glycans are highly branched with several mannoses (hyperglycosylation). Bacteria are also inefficient because of disulfide bond formation, which is guaranteed not only to be formed but also properly crosslinked in mammalian cells. Furthermore, mammalian cells add a sialic acid in the penultimate galactose residue in N-linked glycans, which contribute for protein stability. All these protein modifications are crucial not only for the proper folding but also for protein function and stability (Table II). In this context, mammalian expression systems are the choice for large-scale production of therapeutic peptides that need critical and specific post-translational modifications. The main advantage for expression in mammalian cells is that the recombinant peptides are usually secreted in a soluble form in the cell media. On the other hand, bacterial and yeast systems, although extremely cheaper and easier to manipulate, usually store recombinant peptides in a nonsoluble form, called inclusion bodies⁶³ (Table I). Nowadays, about of 60–70% of all therapeutic peptides are produced in mammalian cell systems, especially in Chinese hamster ovary cells (CHO).¹⁹ Besides its use for large-scale peptide production, this cell line presents a glycosylation pattern similar to human peptides, improving the safety for human health.⁶⁴

To maintain therapeutic recombinant peptides stable for commercial use, some additives are added in the formulations, which can be derived from human or animal sources, including blood, skin, hair, bones, milk, and tendons.⁶³ Commonly, cells are grown in a serum-based medium rich in growth factors and supplements that are important for peptide production. Nevertheless, serum contains some constraints that delay and hinder biopharmaceutical production. However, the use of mammalian cells has raised concerns related to the “mad cow” and viral diseases, which closely relates to pathogen transmission through the serum. Therefore, the development of plasma-free systems is under extensive studies to minimize the risks of diseases and immunogenicity transmission.⁶³

In addition, plasma-free manufacturing demands the elimination of plasma derivatives—divided in several steps—as well as postproduction tests, which guarantee that recombinant peptides are properly folded and preserved. The substitution of supplements that mimic the effects of serum components has become a good choice for plasma-free peptide production. For example, the use of peptide hydrolysates derived from wheat, soy, and yeast contain essential elements that are able to maintain cell viability and peptide productivity.⁶⁵

Unfortunately, the increasing complexity of processes involving the manipulation and security of mammalian cell systems can lead to expanded costs of recombinant peptides production. The main concern, therefore, is whether bio-

pharmaceutical industries will continue to make efforts and apply the safest technologies for mammalian cell lines, or if the introduction of another heterologous system will be more reliable and cost-effective.

BIOREACTORS: TYPES AND APPLICATIONS

Plant Bioreactors

The first demonstration of plant cell cultivation outside the whole plant was done by Haberlandt in 1902 with his article entitled “Attempts of cultivation with isolated plant cells.” Later on, he became the founder of plant cell culture system. Further investigations sedimented the improvement of plant growth *in vitro*, such as changes on temperature, aeration and agitation conditions, culture solutions, and nutrient medium. Hence, the first patent on plant tissue cultivation was granted to Routien and Nickell (1956), in association with Pfizer and Co. Inc. (New York, USA). With the advances in biotechnology and the application of *Agrobacterium tumefaciens* for transferring foreign genes into other organisms, an increase of plant cell culture systems was observed. Consequently, new strategies for cell cultivation were introduced for the production of different compounds in large scale—such as recombinant peptides, plant-derived metabolites and polysaccharides. The design of plant bioreactors enabled a high-scale and high-value production of molecules important for drugs manipulation.⁶⁶

Nowadays, it is possible to induce callus cultures from almost any plant species, especially dicotyledons. For example, the German Collection of Microorganisms and Cell Cultures has around 700 plant cell lines from 80 different plant families in stock (www.dsmz.de). In this context, one important measure for biotechnological production of molecules of interest is the transferring of biosynthetic processes from shake-flasks to bioreactors. Although this transfer is able to preserve the biosynthetic potential of plant cell cultures, it is dependent of various inter-related factors, such as the method of cultivation and physiological features of the plant.

Plant bioreactors have been optimized from prior technologies developed, originally, for microorganisms.^{66,67} Recently, many different bioreactors configurations are used, including the traditional ones—stirred tank reactors, airlift reactors, and bubble column reactors—and new techniques using a cascade of reactors or a combination of diverse reactors.^{68–72} Furthermore, to optimize the production of desired compounds, validate the results and reduce the costs, novel bioreactors for plant cell cultures have been developed. This includes the life reactor, ebb- and flow-bioreactor, platelined

bioreactor, wave bioreactors, slug bubble bioreactor and a Nestlé's wave and undertow bioreactors.

In Wave bioreactors, the circular agitation is replaced by a wave movement, which guarantees not only the proper agitation but also bubble aeration. Therefore, this system is appropriate for shear-sensitive cells cultivation.^{69,70} It consists of using a plastic bag instead of glass cylinders, avoiding the need of sterilization, once that the bags are discardable. The filters permit that media, inductors and bacteria be applied inside the bags without contamination. They can also be used to connect and control oxygen and carbon dioxide concentrations within the media, optimizing the process of cell mass growth. Moreover, the temperature of the equipment, as well as the angle and speed of shaking, can be all monitored by a hand controller. Because of the disposable plastic chambers, the isolation of the culture and the fact that the scaling is done in a laboratory environment, it conforms to the "current good manufacturing practices (cGMP)" regulations.^{69,70} Recently, a group from Nestle R&D Centre in Tours, France, launched two types of bioreactors: (i) wave and undertow and (ii) slug bubble bioreactors. The first one is based on a wave/undertow motion that facilitates bubble-free aeration and mixing of the culture. It can be used for up to 750L culture and was able to increase 1.3-fold the mass when compared with a stirred tank reactor.^{71,72} The second one consists of a plastic cylinder in which the aeration is obtained via generation of large cylindrical bubbles moving from the bottom to the top of the reactor. This bioreactor was efficient for a culture up to 175L and produced a biomass 1.2-fold than the stirred tank reactor.^{71,72}

Algae Bioreactors

Although algae are classified into the plant kingdom, they are considered a unique class of organisms in biotechnology. Algae bioreactors have been widely used for different purposes, such as peptide expression and development of bioenergy sources through light supply and biomass conversion. Eukaryotic green algae were first introduced as an alternative strategy for improving foreign peptide production through chloroplast transformation. Hence, they present many advantages over other peptide expression systems, including the ability of expressing multiple genes in a single transformation event, also leading to a high content of the recombinant peptides.^{73,74} Moreover, the presence of numerous chloroplasts in algae, increase the yield of foreign peptide production and chloroplasts aid in peptide folding performing disulfide bonds, allowing the production of many vaccine antigens and biopharmaceutical products.^{75–77}

Among the recombinant peptides obtained through algae chloroplast, there is a bioactive bovine mammary-associated

serum amyloid (M-SAA), a tumor necrosis factor-related apoptosis-including ligand (TRAIL), the enzyme glutamic acid decarboxylase-65 (GAD65), a peptide E2 of classical swine fever virus and a goat-and-mouth disease virus VP1 peptide.^{65,74,78–80} Currently, the best known and most widely used algae for peptide expression in bioreactors is *Chlamydomonas reinhardtii*.

Furthermore, algae photobioreactors are the system of choice for increasing biomass in a straight-forward, low-cost and efficient way. The effectiveness of photobioreactors can be determined by the light usage, capturing and distributing it among the algae culture.^{32,81} Nowadays, photobioreactors present an automatic monitoring system for the control of media temperature, pH, oxygen, and carbon dioxide, as well as for cell mass growth. Furthermore, the data of cell growth and media conditions can be directly stored and analyzed *in silico*, using specific computer Softwares.

Earlier reports described the construction of a Green Solar Collector (GSC) for microalgae cultivation on sunlight. This system offers flat-panel compartments from where light can be absorbed, well delivered and distributed.^{82,83} Consequently, the combination of decreased sunlight intensities with proper mixing of the algae liquid phase could induce high yields of biomass production.⁸³ Antibodies can also be produced in microalgae systems. Hence, the monoclonal human IgG antibody against the Hepatitis B surface protein was recently developed in *Phaeodactylum tricornutum* cells for future large-scale production of vaccines. It demonstrated that algae cells are not only a good option for human antibody expression but also it is an efficient low-cost alternative, presenting a CO₂-neutral system that allows fast growth rates without the risk of pathogenic contaminations observed in animal cell lines.⁸⁴

Recently, studies reported the use of *C. reinhardtii* photobioreactors along with the yeast *Saccharomyces cerevisiae* for the production of bioethanol.⁸⁵ Microalgae are very effective in CO₂ fixation and release of O₂, as well as for starch accumulation at a high growth rate (Hirano et al., 1997). For this reason, a system was developed using *C. reinhardtii* photobioreactor for the hydrolysis of starch into simple sugar molecules, joined with culturing of *S. cerevisiae* to ferment such sugar into ethanol.⁸⁵ These combined technologies explicit the individual potential of each system and how these systems can be combined to increase production and effectiveness.

Moss Bioreactors

Moss bioreactors came out as a novel and straight-forward alternative for large-scale production of foreigner peptides, especially for biopharmaceutical products development. Mosses contain a small genome of 511 Mbp, which is easier to manipulate than higher plant systems, and it is the only

know plant system to perform homologous recombination. Moreover, the risk of contamination with human and animal pathogens is reduced, when compared with bacteria and mammalian cell cultures. The moss *Physcomitrella patens* is the most widely used system in bioreactors, where the cells are transformed via PEG-mediated protoplast transfection and, provided that they are photoautotrophic, grown in a media composed of inorganic salts, without the need of complex carbohydrate or amino acids sources.²⁵ The advantages of *P. patens* in comparison with seed plants include the high rates of homologous recombination in its nuclear DNA, providing straight-forward reverse genetics protocols by gene targeting.^{71,72,86,87} This also allows targeted knockouts of genes, being extremely useful for production strain design.^{88,89} Furthermore, *P. patens* is a model organism for physiological studies, provided that its growing stages are well-defined and distinct, providing a unique possibility for the analysis of its environmental and development conditions.^{90,91} There is also the fact that mosses major development stage consists in the haploid gametophyte form. Therefore, it makes glycosylation procedures easier to work with, in comparison with other cell cultures.^{32,87} Another advantage of using moss bioreactors is the lack of a significant bias for codon usage, enabling the adaptability of any peptide production.⁹²

Cultivation of *P. patens* is relatively simple and low cost. *In vitro* cultivation of tissues is the best option for mosses and involves the germination of the haploid spore producing protonema that proliferates via apical cell divisions (tip growth). Hence, subsequent divisions of the protonema will stimulate the development of branches or buds, which will be transformed into the gametophore and, finally, the adult plant.²⁵ The formation of sex organs can lead to self-fertilization and the growth of a spore capsule on the top of the gametophore. These spores are later released, completing the life cycle of the organism.²⁵ The medium is made essentially by water and inorganic salts and, although many compounds were tested for their effects on moss growth, they do not require any hormones or complex additives for cultivation.^{93,94} Light and carbon dioxide are the only energy and carbon sources needed. In addition, this organism can grow under a broad range of pH and temperature.^{92,95} Cultivation in glass bioreactor has already been established and, compared with animal cell systems, it is much better adapted to extensive process technology development.⁹⁶

The use of *P. patens* cultures were launched in bioreactors due to the ability of producing different heterologous compounds, provided that the moss can be kept in the protonema stage, and these protonema suspensions can be used for the isolation of protoplast and large-scale transforma-

tion.^{91,97} The fact that mosses are able to perform post-translational processing of peptides, including glycosylation and disulfide bond formation, also made them an excellent tool for the production of biopharmaceuticals and biotechnologically interesting products.⁹⁸ Among many promoters tested from plant, animal and even from *Physcomitrella* expression systems, it was showed that upstream sequences of the moss actin homologue PpAct5 was able to cause the strongest transcriptional activation, as well as the auxin-inducible GH3 and DR5 promoter systems.⁹⁶ Furthermore, secretion signals are useful tools for directing target peptides to the extracellular space, especially when producing complex modified macromolecules. Earlier studies described that not only plants and mosses signals are well interpreted, but human signals can be used for the secretion of recombinant products.^{99,100} Therefore, the use of genetic engineering and optimization of culture conditions allowed successful improvements of product yields into moss systems. The development of targeted gene replacement and use of improved antibodies that function via glycan optimization systems, make moss bioreactors the major alternative method for biopharmaceutical peptides expression.

HUMANIZATION OF PRODUCTS

One of the greatest challenges in producing pharmaceutical products in any recombinant system is the replication of the post-translational modifications that accounts not only for the proper peptide folding, but also for peptide function, stability and turn-over. For instance, only animal cells and transgenic animals and plants are able to perform the correct disulfide crosslinks the light and heavy chains of antibodies.¹⁰¹ Therefore, a continuous attempt to circumvent this issue is the manipulation of the secretory and peptide modification pathways of the diverse expression systems described previously to accomplish the “closest heterologous mimic” of the human interest peptide.

The humanization of insulin and its expression in bacterial systems was the first large-scale production of a biopharmaceutical molecule. It consisted of humanizing the porcine insulin, which diverges from human insulin by only one amino acid residue. This process increased the production of insulin, avoiding the use of animal cells, which also eliminated the risk of virus contamination.^{102,103} Production and secretion of human derived peptides have been widely studied in yeast systems, such as *P. pastoris*. Optimization of the glycosylated bile-salt stimulated lipase (BSSL) production have achieved secretion levels of 1 g/L of culture.¹⁰⁴ However, the obvious differences in glycosylation patterns observed between the yeast and human cells prevent the use of these heterologous peptides for human use.

The manipulation of yeast and insect cells to produce recombinant human peptides with therapeutic purposes confronts the narrow bottleneck of allergenicity caused by species-specific sugars added in the glycosylation process, and the stability of the products which is guaranteed by a terminally linked sialic acid in the case of mammalian cells (including humans).¹⁰⁵ Thus, genetic manipulation studies of eukaryotic cells aiming the reproduction of the complex sequential steps in the human glycosylation pattern have yielded in cell lines capable of specific modifications. For instance, previous studies demonstrated that the knocked-out of four yeast-specific glycosylation genes and the introduction of several heterologous genes have accomplished more than 90% terminal sialylation in the heterologous peptides, which has been claimed to be the most complex step of human glycosylation.⁹⁶

Since the first description of antibody production in tobacco,¹⁰⁶ plants have long been shown to be able to reproduce the complexity of proteins such as antibodies, which depends on several disulfide bridges to attain the right fold.^{106,107} This kind of peculiarity makes plants an interesting source of complex recombinant proteins. However, to our knowledge, all plants species add a $\beta(1,2)$ -xylose and an $\alpha(1,3)$ -fucose in the N-linked glycans, and these plant specific sugars have been shown to be highly immunogenic.^{108,109} There are a few “glycoengineering” strategies to overcome the addition of these allergenic sugars and adapting heterologous protein production in plants:¹⁰¹ (i) elimination of glycosylation sites through site specific mutagenesis, removing of mutatin Asn or Ser/Thr residues that are prone to be recognized by the glycosylation machinery in the ER. This approach is the most drastic due to its amenability to cause misfolding and consequently activity and longevity problems to the product; (ii) inhibition of Golgi glycosyltransferases, for the complex glycosylation pattern is achieved at the Golgi apparatus rather than in the ER^{110,111}; (iii) addition of ER retention signals (H/KDEL) with the rationale of preventing the proteins of reaching the Golgi apparatus, where the complex sugars are added^{112,113}; (iv) overexpression of human glycosyltransferases in plants used as biofarming platforms; and (v) the expression of the enzymes responsible for the addition of terminal $\beta(1,4)$ galactose and the subsequently addition of a sialic acid to this galactose residue in N-glycans, provided that the sialic acid is essential for intravenous biopharmaceuticals due to its key importance in its half-life (plasma proteins lacking the glycan terminal sialic acid have increased clearance rates).^{114,115}

Glycoengineering studies have been carried out in the moss *P. patens*, taking advantage of its homologous recombination. Hence, plant specific sugars, such as fucose and

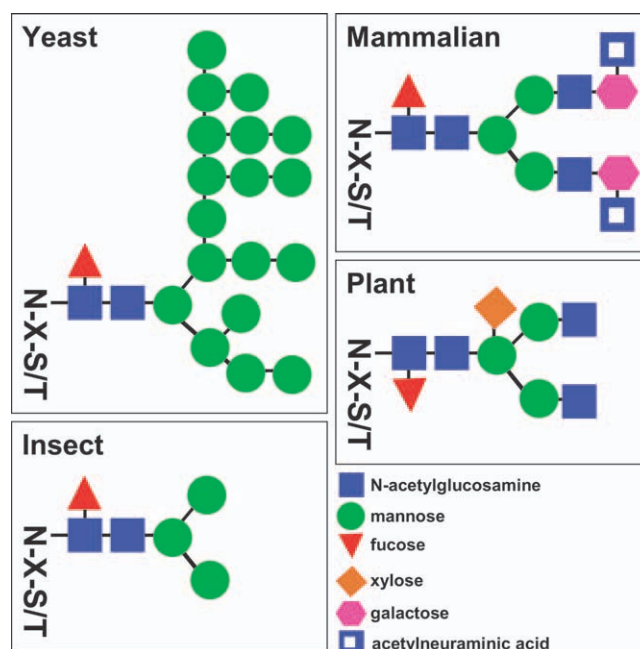


FIGURE 1 Different glycosylation patterns produced by diverse cell types. It is noteworthy that the fucose in mammalian glycans is in an $\alpha(1,6)$ ligation, whereas in plants, the same fucose is bound to N-acetylglucosamine in a $\alpha(1,3)$ conformation. This is a potential allergenic feature that is removed in the N-glycan knock-out moss line produced by Koprivova et al.,¹¹⁸ as well as the removal of the plant specific xylose. These modifications produce an incomplete (when compared with mammalian) glycosylation pattern, however functional and nonallergenic.¹¹⁷ Adapted from Ma et al.¹¹⁹ and Gerlach et al.¹²⁰

xylose, were removed from human peptides expressed in the moss by targeted knock-outs to the sugar adding glycosyltransferases^{116–118} (Figure 1). This line was shown to grow well in cultures without any observable phenotypic difference, and without changes in secretion ability, which makes it an excellent tool for the production of humanized and nonallergenic peptide products for human use.

CHLOROPLASTS AS POTENTIAL BIOFABRICS AND DEPOSITS OF HETEROLOGOUS PEPTIDES

Heterologous peptide expression, in any system, is influenced by several factors, which range from: the exogenous gene insertion in the genome; the proper induction of gene expression by choosing the right promoters and regulatory sequences; the appropriate folding and post-translational modifications; and subcellular targeting and/or secretion. To avoid transgene insertion issues in plant expression systems, the moss is an excellent choice due to its homologous recombination, which prevents insertion in undesired loci, main-

Table III Biopharmaceutical Compounds Produced into Heterologous Systems

Product	Commercial Name	Use of Product	Cell System Used for Production	Year When Commercialization Started	References
Insulin	Humulin	Diabetes	<i>E. coli</i>	1982	133
IX factor	Benefix	B Hemophilia	CHO	1997	134
β -interferon	Avonex	Multiple sclerosis (MS)	CHO	1996	135
VIII factor	Refacto	A Hemophilia	CHO	2000	136
MAb	Simulect/basiliximab	Organ rejection	Sp2/0	1998	137
MAb	Campath	Leukemia	CHO	2001	138
MAb	Avastin/bevacizumab	Colorectal cancer	CHO	2004	139
MAb	Xolair/omalizumab	Asma	CHO	2003	140
MAb	Soliris/eculizumab	PNH	NS0	2007	141

MAb: monoclonal antibodies; CHO: cells from human ovary; NS0: mammalian cell lines from hybridoma cells; Sp2/0: myeloma cells; PNH: polynitroxyl-alpha-hemoglobin.

taining the inclusion targeted to a specific locus.^{91,93,97} A recent report has also shown a new strategy to accomplish this kind of transgene specific targeting in maize, which relies on a double-stranded break at specific target loci induced by designed zinc-finger nucleases.¹²¹

Indeed, several factors influence the efficiency of an expression system, such as peptide instability and degradation.¹²² One approach to circumvent the instability issue is to physically protect the expressed peptides in cell compartments such as plastids. Chloroplast expression of foreign peptides has been recently developed and successfully used for high level production of peptides with diverse complexities.^{76,77,123–126} Gene silencing and positional effects of transgene insertion can be avoided in chloroplasts.^{127,128} Moreover, the accumulation of the peptide of interest is granted by the number of plastids in a single cell, which varies from a couple to thousands, each single one containing a copy of the transgene and potentially expressing the desired peptide.

Chloroplast expression may also be an alternative for another challenge in peptide expressions systems yet to be fully addressed: the expression of highly hydrophobic transmembrane peptides. Insights on this issue have been raised in recent studies that overexpressed a chloroplast translocon peptide from *Arabidopsis thaliana* (AtTic40) in tobacco via plastid transformation.^{126,129} AtTic40 is a single transmembrane peptide helix¹³⁰ and it has been reported to accumulate in such high levels in the organelle that the chloroplast inner-envelope membrane expands and folds several times within the organelle, suggesting an increase lipid and peptide components for accommodation of enhanced accumulation of Tic40.¹²⁹

Recent studies have brought to light targeting determinants for chloroplasts membranes.^{131,132} The ability of chlor-

oplasts to synthesize and expand its inner-envelope membrane without any significant effects on plant phenotype or productivity,¹⁰⁷ enlightens the utilization of chloroplasts as bioreactors for expression and/or accumulation of transmembrane peptides with further biotechnological and pharmaceutical applications.

CONCLUSION AND PERSPECTIVES

In this report, we observe the availability of a wide assortment of possibilities for recombinant peptide expression from many different sources (bacteria, plants, mammals, etc). In the past years, the technology development applied to each expression system has provided the opportunity of production of different peptides using a single system (Table III). Hence, the choice for the right expression cell, vector, culture medium and application of bioreactors depends not only on the adequacy of a certain expression system but mostly on its ability to offer specific characteristics for the desired peptides. Some systems contribute for a better quality on peptide production than others, yielding molecules with identical configurations as in its original organism. These are extremely useful when the structure of the recombinant peptide is important for its best function. Moreover, the use of chloroplasts and other organelles are interesting systems when a large amount of peptide deposit is needed, without significant effect or toxicity on the cell itself. To date, even the most complex expression systems are becoming more accessible and straightforward to work with, provided that most of expression vectors, culture media, and other key components are now easily available in the market, as well as several protocols for specific peptides production optimization.

In addition, the improvement of biotechnological equipments for large scale peptide production resulted in several

different types of bioreactors, each of them containing their own features and applicability. Whether the purpose is to produce biopharmaceutical molecules or to explore the sources for bioenergy technology, bioreactors have become a strategic alternative to obtain a good quality of high cell mass levels in a relatively short time period. Together with the right expression system, recombinant peptide production in bioreactors has reached its highest level in terms of efficiency, economy, and excellence. Therefore, as long as new peptides are described in nature, there will be different expression systems being developed, optimized and applied in biotechnology.

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