Biopharmaceutical production via moss bioreactors

The moss bioreactor has a number of unique properties which make it superior to other cell systems – animal cells, higher plants, bacteria, fungi – in the production of biopharmaceuticals.

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Current production of complex biopharmaceuticals relies mainly on animal cell systems such as the CHO-cell. Plant systems show major safety and cost advantages over processes based on animal cells, but differences in glycosylation as well as environmental release are factors that may give rise to concerns. Moss tissue can be cultivated in liquid culture and, as such, cultivation is achieved in a contained system. Glycosylation patterns on proteins produced in mosses can be engineered to be ‘human-like’. Thus, the moss bioreactor as a contained plant tissue-based cultivation system combines the best of two worlds.

greenovation’s moss bioreactor is based on the fermentation of moss protonema (Figure 1). This fully differentiated tissue is extremely stable over time (for example, several months of cultivation) and is highly homogeneous since it is composed of only two cell types. The moss bioreactor has further unique properties:

- The moss tolerates humanisation of the glycosylation pattern through homologous recombination,
- Time-to-market is comparable with traditional systems. A transient expression system allows feasibility studies to be undertaken, and stable production strain development takes only four to six months,
- Cultivation in suspension allows for the scale-up of moss photobioreactors up to several 1000 L, and
- Through phototrophic cultivation, a simple medium can be utilised which is non-hazardous. This, in combination with secretion of the heterologous protein of interest, greatly facilitates downstream processing.

**Proof of concept**

The moss, *Physcomitrella patens*, has been a model organism for physiological and genetic research for more than 30 years. Much EST (expressed sequence tag) data are available on the internet and in various publications. *Physcomitrella patens* has a comparatively small genome (511 mbp) and it is a haploid organism.

For proof of concept purposes, *Physcomitrella* was transformed to express human VEGF (vascular endothelial growth factor); production and secretion
Western blot analysis under non-reducing conditions of moss derived transiently expressed rhVEGF with different leader peptides: 1) Control 5ng, 2) 10ng, 3, 4) Different leader peptide.

Moss is the only known plant system which shows a high frequency of homologous recombination. This attribute allows for targeted gene insertion leading to the stable integration of foreign gene(s).

Humanisation of the glycosylation pattern

Compared with bacteria, mosses are far more complex organisms and display more sophisticated post-translational modification capabilities. Because of the inherent limitations of bacterial and mycotic systems, many proteins cannot be expressed in such organisms in a functional form.

Glycosylation patterns in mosses are the same as for the higher plants. This means that the core structure of N-glycosylation is identical to the typical bi-antennary structure found in animal cell fermentation systems. The differences are two additional plant-specific residues and the lack of terminal galactose residues. The additional beta 1-2 linked xylosyl and alpha 1-3 linked fucosyl-residues found in plants are generally associated with an allergenic potential. Removal of these residues is therefore relevant, especially for parentally administered drugs.

Moss is the only known plant system which shows a high frequency of homologous recombination. This attribute allows for targeted gene insertion leading to the stable integration of foreign gene(s). Another important aspect of the moss system is that it can be manipulated to make targeted gene knockouts. Homologous recombination is therefore a highly attractive tool for production strain design.

The xylosyl- and fucosyl-transferases can be knocked out resulting in removal of these sugar residues, as confirmed by Maldi-TOF analysis. Through this, the plant-specific glycosylation pattern is manipulated while retaining glycosylation patterns that are highly similar to those found in animal cells. Major parts of this work have been performed in cooperation with Professor Reski at the University of Freiburg (Germany). These knock-out mutants have been analysed at the physiological level and also for the secretion of heterologous proteins. No differences could be found relative to moss strains with a wild-type glycosylation pattern.

Another difference in plant glycosylation patterns, as compared with animal structures, is the lack of a fucosyl-1,6-residue in plants. No pharmacokinetic effects are related to this sugar residue, so no allergenic potential is expected as a result of this missing sugar residue. In addition, a decrease in the amount of this sugar residue significantly increases antibody-dependent cellular cytotoxicity (ADCC) of recombinant antibodies. It is concluded that this missing sugar in plants may prove to be an advantage.

Terminal galactose residues are not usually associated with protein activity, but they may have a pharmacokinetic effect – and so the lack of these residues needs to be engineered. Should the galactose residue be relevant for the final product, the appropriate galactosyltransferase should be inserted. Galactose is available in the organism, so that this single insertion is sufficient to ensure galactosylation. Data from higher plants have shown that this modification has no influence on plant viability.

By carrying out these steps – that is, knock-out of two plant-specific transferases and insertion of one new transferase – the engineered moss tissue is able to adopt a glycosylation pattern to the antibody protein of interest typical for the production of monoclonal antibodies.

Time-to-market

Time-to-market for biopharmaceuticals produced via the moss bioreactor is comparable with traditional systems. A transient expression system allows feasibility studies to be undertaken, and stable production strain development does not require crossing steps or the regeneration of whole plants.

The transient system is based on transformation of moss protoplasts (Figure 3); the system enables production of milligram quantities for feasibility studies within several weeks. Protoplasts are obtained from protonema cultures and cultured in 96-well-plates for 96 hours producing significant amounts of the protein of interest. Cultivation in 96-well-plates also allows for automation of major elements of the process.

Production of a stable expression strain takes four to six months. This process also leads to strains containing multiple copies of the heterologous gene, so that the optimal production strain can be directly selected from a first pool of transformants.

Figure 2 Western blot analysis under non-reducing conditions of moss derived transiently expressed rhVEGF with different leader peptides: 1) Control 5ng, 2) 10ng, 3, 4) Different leader peptide.

Figure 3 Moss protoplasts.
The yield of heterologous protein – using the model protein, human VEGF – was expected to reach 30 mg per litre per day by the fourth quarter of 2003; this corresponds to the yield of a typical fed-batch culture system over 20 days of 600 mg per litre. The typical process for the moss bioreactor will be a perfusion system, since the moss system is stable for long periods of time (>180 days). The risk of contamination with the moss system is extremely low.

Further increases in yield will be achieved by the optimisation of production strains for specific proteins. As downstream processing involves few purification steps, yield loss downstream is reduced compared with animal cell systems.

**Safety profile**

Moss is photo-autotrophic and therefore only requires a very simple growth medium compared with the complex media needed for animal cell systems. Costs are thus reduced and media contamination risks are minimised. There are no moss viruses known and there are no known plant viruses that could be pathogenic to humans.

Antibiotics are not needed for the cultivation of transgenic moss. Transgenes are integrated into the genome and contamination of the medium is highly improbable. Thus, the risk of having trace amounts of antibiotic residues present in the final product is avoided. Furthermore, in this contained system, there is no need to address environmental issues that may be connected with the release of transgenic plants into the environment.