

## THE FUTURE OF BIOPHARMACEUTICS' PRODUCTION

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Regarding the augmented pace of protein research and market demand, more efficient and rapid expression of genes in homologous and heterologous expression systems must be developed. All expression systems offer advantages and disadvantages, thus the proper choice of a host organism for the production of a biopharmaceutical has to be made on a case-by-case basis and depends upon the protein under consideration. Here we review some major expression systems used in biopharmaceutical industry.

### 1. Introduction

During the last years many biopharmaceuticals were approved clinical trials and commercial production resulting in a market of about US\$50 billion in 2005. It is also estimated that 400–500 biotech drugs are under clinical development for various disease conditions. Estimated 4,400 biotech companies are globally involved, having brought more than 100 different biopharmaceutical products on the (U.S.) market [1].

Depending on required characteristics, biopharmaceuticals are produced in different host systems. Although transgenic plants and animals are predicted to become commercially competitive for manufacturing therapeutic proteins in the future, every biopharmaceutical on the market today is produced in either a mammalian or microbial cell line [2]. As a result over half (55%) of such products are expressed using microbes, either bacteria (40%) or yeasts (15%), with nearly all those bacteria being a form of *E. coli* (39%). Another 45% are expressed in mammalian cells primarily CHO cells (35%) [3].

The development of the protein and its laboratory-scale production is just the beginning for a biotechnology company with ambition beyond discovery. Taking a biomanufacturing process from laboratory to clinical production can be a precarious journey, littered with a multitude of possible pitfalls, some of which could prove fatal.

Expression systems are generally patented and available for licensing from their owners or from companies having licensed the patents for bundling (sublicensing) with their own expression systems or

components [3]. Here we try to compare some specifications of major expression systems and help you to predict the growth procedure of expression systems in biopharmaceutical industry.

### 2. Bacteria

Expression of recombinant proteins in *E. coli* has been investigated extensively over the last 20 years and is one of the most frequently used systems. Clear guidelines by the competent authorities (e.g. EMEA and FDA) for the production of recombinant proteins in *E. coli* have to be complied [4]. Some of today's blockbusters are expressed in *E. coli* (table1) because it can be easily scaled-up in high cell-density fermentations yielding protein accumulation levels in the gram per liter scale [5].

An additional benefit is the established rapid cloning and expression procedure. With such technology, expression levels have reached in excess of 50% of total cell protein [6]. Small polypeptide tags, including the hexahistidine (his-tag), the FLAG or the anti-c-myc tag are added either N or C terminally to the protein for detection and purification [7]. Employing leader sequences (e.g. pelB or ompA leader), recombinant proteins are directed into the bacterial periplasm, where the oxidising milieu favours the formation of disulfide bonds by the disulfid-oxidoreductase [8]. Another advantage is the presence of chaperones leading to stabilisation of the recombinant protein. Overexpressed proteins often fail to fold correctly and undergo proteolytic degradation [9].

## 2.1. Advantages

Established regulatory track record; well-understood genetics; cheap and easy to grow; inexpensive media; high expression levels quickly — sometimes within five days; fast-growing (growth time measured in minutes); easy characterization (with few adventitious agents) [10].

Table 1. Biopharmaceuticals expressed in bacteria.

| Generic               | Brand name  | Company              |
|-----------------------|-------------|----------------------|
| Anakinra              | Kineret     | Amgen                |
| Filgrastim            | Neupogen    | Amgen                |
| Insulin Glargine      | Lantus      | Aventis              |
| Insulin Human         | Humulin     | Eli Lilly            |
| Insulin               | Lispro      | Eli Lilly            |
| Interferon alfa-2b    | Intron-A    | Schering Corporation |
| Interferon Beta-1b    | Betaseron   | Berlex Corporation   |
| Nesiritide            | Natrecor    | Scios Inc.           |
| Oprelvekin            | Neumega     | Wyeth                |
| Pegfilgrastim         | Neulasta    | Amgen                |
| Peginterferon Alfa-2a | PEGasys     | F.Hoffmann-La Roche  |
| Peginterferon alfa-2b | PEG-Intron  | Schering-Plough      |
| Retoprase             | Retavase    | DL BioPharma         |
| Somatropin            | Genotropin  | Pharmacia            |
| Somatropin            | Humatrope   | Eli Lilly            |
| Somatropin            | Norditropin | Novo Nordisk         |
| Somatropin            | Nutropin    | Genentech            |
| Teriparatide          | AQ Forteo   | Eli Lilly            |

## 2.2. Disadvantages

Proteins are not usually secreted (so cell disruption step complicates harvesting); contain endotoxins; are microheterogeneous; no posttranslational modifications (no glycosylation); possibility of incorrect protein folding; harvesting can damage proteins [10].

## 3. Yeast

Yeasts are recognized by a growing track record as expression platforms for the production of pharmaceuticals. Yeast expression system includes lower eukaryotes like yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and methylotropic yeasts like *Pichia pastoris*, and *Pichia methanolica* have gained importance. They are used in large-scale fermentation processes for beer and other products for hundreds of

years (table2). Nowadays the cost of recombinant protein production in yeasts usually runs between \$50 and \$100 per gram of final product.

Vectors:

YIp Vectors: do not replicate autonomously, but integrate into the genome at low frequencies by homologous recombination.

YEep Vectors (yeast episomal plasmid): replicate autonomously because of the presence of a segment of the yeast 2  $\mu$ m plasmid that serves as an origin of replication.

YCp Vectors (yeast centromere plasmid): replicate autonomously and contain centromere sequences (CEN), and autonomously replicating sequences (ARS).

Selection Markers:

Dominant selection markers: antibiotic markers such as G418 and cyclohexamide

Complementation markers: marker genes that complement an auxotrophic mutation in the genome like URA3, TRPI, HIS3, and LEU2.

Promoters:

Inducible: like ADH2, SUC2

Constitutive: like GAPDH

Secretion Signals: Prepro alpha factor, HSp150, PHO, SUC2, KILM1 (killer toxin type 1), GGP1

Table 2. Biopharmaceuticals expressed in yeast.

| Generic        | Brand name | Company      |
|----------------|------------|--------------|
| Becaplermin    | Regranex   | Ortho-McNeil |
| Glucagon       | Glucagen   | Novo Nordisk |
| Insulin Aspart | Novolog    | Novo Nordisk |
| Insulin Human  | Novolin    | Novo Nordisk |
| Lepirudin      | Refludan   | Berlex       |
| Sargramostim   | Leukine    | Berlex       |

## 3.1. Advantage

“Generally recognized as safe” by regulators; long history of use; genetics well understood; no endotoxins; high expression levels fairly quickly (two to eight weeks); protein is secreted for easy harvesting; fast growth (hours); inexpensive media; proteins usually properly folded; posttranslational modifications [10].

## 3.2. Disadvantage

Overglycosylation can ruin protein bioactivity, safety, activity, potency, or clearance; can contain immunogens

or antigens; nonnative proteins are not always properly folded [10].

#### 4. Insect cells

Protein production in insect cells has become more and more relevant for research and development applications (table3). Insect cells are easy to transfect with baculovirus vectors to yield high amounts of protein — with post-translational modifications which are quite close to, but not as complex as, in human systems. Novavax Company work in this filed.

Table 3. Some insect cell lines.

| Cell line          | Source                               | Cell density in culture  |
|--------------------|--------------------------------------|--|
| Sf9                | Spodoptera frugiperda ovarian tissue | $>12 \times 10^6$ cells/ml in suspension on day 6-7 with $>98\%$ viability |
| Sf21               | Spodoptera frugiperda ovarian tissue | $>6 \times 10^6$ cells/ml in suspension on day 6-7 with $>98\%$ viability  |
| Tn5B1-4(high five) | Trichoplusia embryonic tissue        | $>8 \times 10^6$ cells/ml in suspension on day 6-7 with $>98\%$ viability  |
| S2                 | Drosophila                           | $> 50-100 \times 10^6$   |

##### 4.1. Advantages

Posttranslational modifications; properly folded protein; product is secreted; fairly high expression levels; expresses within about four weeks; baculoviruses are harmless to humans [10].

##### 4.2. Disadvantages

Minimal regulatory track record and short history of use; slow growth, expensive media; baculovirus infection is an extra step in the process; contain immunogenic host cell proteins; some incorrect glycosylation; mammalian viruses can infect the cells in warm culture [103].

#### 5. LEXSY

Leishmania tarentolae, a unicellular, flagellated protozoan organism, belongs to a family of parasites shuttling between sandflies and vertebrates. Since isolation from its host Tarentola mauretana in 1921, L. tarentolae is kept in axenic culture, and it is not pathogenic to mammals. The LEXSY expression

strains are therefore grown under S1 laboratory safety conditions.

LEXSY strains can be handled like E. coli: they may be grown on agar plates or in liquid media from 96-well plate up to fermenter format. They have doubling times as short as 4 hours under optimum aeration and grow to high density within short time.

#### 6. Mammalian cells

Since 1986, when Genentech announced the market approval of human tPA, the first therapeutic protein from recombinant mammalian cells, this market has increased potentially to several hundred clinical candidates currently in company pipelines (table4). Sixty to seventy percent of all recombinant protein pharmaceuticals are produced in mammalian cells, as mammalian expression reveals the most authentic glycosylation leading to a high percentage of active complex proteins. Some of these cell lines are CHO, NS0, BHK, HEK-29 and PER-C.

Extensive studies have yielded mammalian cell productivity in bioreactors to the gram per litre range (corresponding to a specific productivity of 20 - 90 pg/cell/day), which represents a more than 100-fold yield improvement in the last 25 years. Employing leader sequences (e.g. Ig kappa leader), allows expression of the recombinant protein into the culture supernatant facilitating the purification procedure. Correct folding and functionality of the recombinantly produced proteins is achieved by chaperones and disulfidomerase located in the ER.

##### 6.1. Advantages

Usually fold proteins correctly; usually make correct posttranslational modifications; can secrete protein; good regulatory track record; only choice (except for transgenics) for the largest, most complicated proteins [10].

##### 6.2. Disadvantages

Expensive media; slow growth; may contain allergens and contaminants from bovine sources; require extensive characterization; complicated purification; expensive, \$500–5,000/gram of final product, very time and labour intensive to reach stable clones [10].

Table 4. Biopharmaceuticals expressed in mammalian cell culture.

| Generic           | Brand name     | Company                   |
|-------------------|----------------|---------------------------|
| Abciximab         | Reopro         | Centocor, Inc & Eli Lilly |
| Abciximab         | Reopro         | Centocor, Inc&. Eli Lilly |
| Alteplase         | Activase       | Genentech, Inc.           |
| Bevacizumab       | Avastin        | Genentech                 |
| Cetuximab         | Erbitux        | ImClone Systems Inc.      |
| cyclosporine      | RESTASIS       | Allergan, Inc             |
| Darbepoetin Alfa  | Aranesp        | Amgen                     |
| Darbepoetin Alfa  | Aranesp        | Amgen                     |
| Dornase Alfa      | Pulmozyme      | Genentech, Inc.           |
| Drotrecogin Alfa  | Xigris         | Eli Lilly                 |
| Efalizumab        | Raptiva        | Xoma, Ltd. & Genentech    |
| Epoetin Alfa      | Epogen/Procrit | Amgen                     |
| Etanercept        | Enbrel         | Amgen& Wyet               |
| Factor VIIa       | NovoSeven      | Novo Nordisk              |
| Factor IX         | Benefix        | Wyeth                     |
| Factor VIII       | Kogenate FS    | Bayer Corp.               |
| Factor VIII       | Recombinate    | Baxter Healthcare         |
| Factor VIII       | Refacto        | Wyeth Europe Ltd          |
| Folitropin Alfa   | Gonal-FREE     | Serono S.A.               |
| Folitropin Beta   | Follistim AQ   | Organon                   |
| Gemtuzumab        | Mylotarg       | Wyeth                     |
| Imiglucerase      | Cerezyme       | Genzyme Corporation       |
| Infliximab        | Remicade       | Johnson & Johnson         |
| Interfron Beta-1a | Avonex         | Biogen Idec               |
| Interfron Beta-1b | Rebif          | Serono                    |
| Natalizumab       | Tysabri        | Biogen Idec& Élan         |
| Omalizumab        | Xolair         | Genentech / Novartis      |
| Palivizumab       | Synagis        | MedImmune                 |
| Rituximab         | Rituxan        | IDEC Pharmaceuticals      |
| Somatropin        | Saizen         | Serono                    |
| Somatropin        | Serostim       | Serono                    |
| Tenecteplase      | TNKase         | Genentech                 |
| Thyrotropin Alfa  | THyrogen       | Genzyme Corp              |
| Urokinase         | Abbokinase     | ImaRx                     |

## 7. Transgenic animals

Securing a safe, economical, and reliable supply of a recombinant therapeutic protein for use in clinical trials and commercialization is an important strategic issue that must be addressed early in the development process.

The construction and validation of cGMP compliant facilities requires typically three to five years, which creates challenges in matching capacity to demand. As a result, companies must make manufacturing choices and

a substantial upfront investment in capacity while their products are still in the test phases. The uncertainty of regulatory approval and the difficulties in predicting market demand impose a significant business and financial risk. For drug developers, investing large amounts of capital on a drug that is awaiting approval is an expensive and very risky prospect; however, having a drug that is approved without a reliable supply of product can have equally dire consequences.

Transgenic production holds tremendous promise for dealing with the cost, capacity and scale-up limitations faced by traditional systems. Some companies involved in this area are PPL therapeutics, Pharming, Genzyme.

### 7.1. Advantages

Capable of complex protein processing and of very large proteins; very high expression levels; correctly fold proteins; easy scaleup; low-cost production (\$20–50/gram of final product) [10].

### 7.2. Disadvantages

Little regulatory experience; unknown potential for viral contamination; variable expression levels; long time scales; unanswered purification questions; continuous production complicates definition of batches and lots; questions regarding observance of cGMPs on the farm; unresolved public image problems [10].

## 8. Avian transgenics

The eggs, from which the proteins are harvested, are natural protein-production systems. But production of transgenic birds is still several years behind transgenic mammal technology. Intensive animal housing constraints also make them more susceptible to disease. Some companies involved in this area are TranXenoGen, Origen Therapeutics, AviGenics.

### 8.1. Advantages

Short gestation and maturation cycles; prolific reproduction; totally low cost for high yield of production; post-translation modification, no human-virus contamination [10].

### 8.2. Disadvantages

High cost for research stage, susceptible offspring to disease because of sterile environment [10].

## 9. Transgenic plants

Commercial production of plant secondary products has been used for centuries in human medicine. The progress of molecular plant genetics, and particularly of transgene expression in plants, has spurred the interest of academia and companies to consider them as eukaryotic hosts for biopharmaceutical production (table5). Two arguments favouring plant hosts have frequently been quoted, namely safety – the absence of adventitious mammalian viral or prionic contamination – and the unlimited scalability of production. The costs of producing recombinant proteins in plants might be 90-fold lower than the production in other systems. Additionally, safe biopharmaceuticals can be produced in plants, since plants do not harvest human pathogens compared to mammalian cells. Some companies involved in this area are CropTech, Prodigene, Boyce Thompson.

Table 5. Transgene expression systems in plants.

| Expression System   | Harvested Biomass                  |
|---|------------------------------------|
| Stable Genome Transformation<br>– Nuclear transformation<br>Leaves, tubers, seeds<br>– Chloroplast transformation | Leaves,<br>tubers, seeds<br>Leaves |
| Transient Expression<br>– Viral transfection<br>– Pro-viral transgene<br>amplification                            | Leaves<br>Leaves                   |
| Zygotic Induction of Transgene<br>Amplification   | Seeds                              |

### 9.1. Advantages

Shorter development cycles than animals; easy storage of seed banks; easy scale-up; good expression levels (up to 1 kg purified recombinant protein/acre of crop); well-understood genetics; no plant viruses known to infect humans (so viral characterization unnecessary); low-cost production (\$10–20/gram of product) [10].

### 9.2. Disadvantages

Potential for new contaminants (soil fungi and bacteria, plant-sourced impurities and metabolites, pesticides, herbicides); posttranslational modifications differ from those made by animal cells; contain possible allergens; unresolved public issues [10].

## 10. Conclusion

Although traditional expression systems have been advantages is indicative of industry-wide problems. Truly novel expression systems such as fungi, insect cells, plants, and transgenic animals have yet to make much impact on marketed products or those in later-stage development. substantially improved over the years, the failure to adopt new technologies often offering considerable

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