Chapter from the book *Innovations in Biotechnology*
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1. Introduction

Biotechnology generates global sales in the order of well over 200 billion US$ in all markets and has thus become an important economic factor in manufacturing. The buzz word ‘biotechnology’ carries expectations that it can provide sustainable solutions for greenhouse gas reduction in manufacturing industries, trigger a ‘clean tech’ boom and create new jobs. It is no wonder that biotechnology has gained significant attention even in high level politics as it can give a ‘green’ touch to administrations. Many consumers are not even aware of the surprising array of products and services which biotechnology can or could provide today; these range from high-tech pharmaceutical applications to snow making. Table 1 lists some new or unusual applications of biotechnology products which include, for example, skin protection compounds from the oceans or biopolymers for drag reduction in transport pipelines.

- Microbial secondary metabolites for the bio-control of invasive mussels in water pipes
- Microbial products for rust removal and anticorrosion
- Proteins for plant protection by induction of the plant’s natural defence systems
- Glycoproteins radically affecting the palate and sensorial perception
- Compounds from deep-sea microorganisms for skin protection
- Nanoscaffolds (also functionalized) based on biomaterial for e.g. tissue replacement and repair
- Biopolymers and biosurfactants for drag reduction in transport pipelines
- Biopolymers and enzymes for plywood production
- Channel proteins for H₂O desalination and other purposes
- Enzymes as toxic gas antidotes for military applications
- Microbially derived innovative lubricants
- Biologicial production of solar cells

Table 1. A few examples of new and unusual applications of biotechnology and its products in different fields.

These high expectations are merited due for 4 reasons:
1. The unmatched precision in the production and assembly of small and large molecules. This precision of the natural biosynthetic machinery cannot be reached using chemical approaches.

2. The fantastic speed, at which these production systems can reproduce themselves. The reason for this is that bacteria have by far the largest surface-to-volume ratio in the living world, leading to maximal metabolic rates. A single bacterium, weighing about $10^{-12}$ grams, grows so fast that its biomass would theoretically reach the mass of the earth in only a few days!

3. The inherent safety of biological systems as metabolic heat makes run-away reactions impossible, when compared to organic chemistry.

4. The biocatalyst and biomass are fully recyclable.

Consequently, biotechnology will have an especially high impact in the production of complex chemicals used for pharmaceuticals, fine chemicals and specialities (Meyer, 2011). Other promising areas are biopolymers and protein-based novel biomaterials for consumer goods, car parts, medical devices or as support for the 2D and 3D cultivation of tissue and organ replacements.

It is industrial or white biotechnology which is of growing academic and private interest, as it represents an equal or even bigger business potential than red biotechnology in the long term. But how can application fields and markets of biotechnology be classified? One way to describe the different markets of biotechnology is the colour code (red, white, green, blue and grey).

2. The markets

A useful way to classify the applications of biotechnology is the colour code of biotechnology shown in Table 2.

Estimates and definitions may vary, but there is one common denominator in various assessments, namely that the proportion of products manufactured using biotechnology will increase significantly. While the development and the market introduction of new biopharmaceuticals such as monoclonal antibodies will continue at its present rate, it is especially industrial biotechnology which is expected to realise high growth rates.

The different “biotechnologies” do overlap and especially the boundaries especially between red and white biotechnologies for pharmaceutical applications can be confusing. There is one important additional difference between the red biotechnology of therapeutic proteins and monoclonal antibodies and the white biotechnology pharmaceuticals which includes a large variety of products: red biotechnology is characterised more by its products whereas white biotechnology is defined more by its technology platform.

2.1 The chemical market

The sales of global chemical markets are expected to grow from 2292 billion Euros in 2007 to 3235 billion Euros in 2015 and to 4012 billion Euros in 2020 (Perlitz, 2008). It is estimated that only about 3-4% of all chemical sales have been generated with some help from biotechnology (Nieuwenhuizen et al., 2009), but this figure is anticipated to grow faster than the average market rate. It is speculated that at least 20% of the global chemicals will be derived using
industrial biotechnology in 2020, which translates into almost 1000 billion Euros. This means that the sales generated by industrial biotechnology will increase by an order of magnitude as the recent estimates of the global sales of industrial biotechnology products vary between 50 and 150 billion Euros, depending on whether biofuels are included or not. There is a consensus that biotechnology will play a much greater role in future manufacturing as it can deliver complex products using economically and ecologically sustainable processes.

<table>
<thead>
<tr>
<th>Markets served</th>
<th>Industrial Biotechnology</th>
<th>Pharma Biotechnology</th>
<th>Environmental Biotechnology</th>
<th>Agro-Biotechnology</th>
<th>Marine Biotechnology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Many different markets such as small molecule pharma &amp; fine chemicals, flavour &amp; fragrance, bulk chemicals etc.</td>
<td>Monoclonal antibodies and other therapeutic proteins</td>
<td>Environmental biotechnology, services, &amp; solution for bioremediation and waste treatment</td>
<td>Transgenic or genetically modified (GM) seeds and plants</td>
<td>Products and lead substances from the marine environment</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Color code</th>
<th>White</th>
<th>Bio</th>
<th>Grey</th>
<th>Green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Market size US$</td>
<td>&gt; 50 bn without biofuels</td>
<td>&gt; 100 bn</td>
<td>n.a.</td>
<td>&gt; 11 bn</td>
<td>n.a.</td>
</tr>
<tr>
<td>CAGR</td>
<td>15%</td>
<td>&gt; 20%</td>
<td>n.a.</td>
<td>15-20%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Companies</td>
<td>4’000</td>
<td>6’000</td>
<td>n.a.</td>
<td>&gt; 50</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 2. Classification of the applications of biotechnology with respect to markets; for each class sales volumes, compound annual growth rate and number of companies globally active in the field are noted; there is of course an overlap between industrial and pharmaceuticals biotechnology, some sectors of industrial biotechnology are dependent on cheap and reliable sources from agro biotechnology (Clive, 2010). The market size of >11 billion US$ for agro biotechnology refers to “seed biotech”. The global value of marketed harvested goods resulting from these seeds would be much larger, by one or two orders of magnitude.

The products potentially produced by biotechnology range from commodities (e.g. succinic acid), biopolymers (e.g. polyhydroxybutyric acid), flavour & fragrance products (e.g. vanillin), agroproducts (Bacillus thuringiensis) to small molecule pharmaceuticals and more.

Unfortunately there are no shortcuts in biotechnology, and in order to meet the anticipated 1000 billion Euros derived from white or industrial biotechnology and to keep red biotechnology humming we need to further develop appropriate tools while keeping in mind that it took 200 years to complete today’s chemical toolbox (Ghisalba et al., 2010).

2.2 The feed, food and dietary supplement markets

Biotechnology plays an important role in the kitchen through the food and beverage industry. Enzymes are used in large scale food production of glucose from starch (hydrolytic enzymes), high fructose syrup (isomerase), conversion of lactose to galactose and glucose (hydrolase), cheese production (proteases), meat processing (proteases) and
many more. Phytase (phosphohydrolase) is an example of an enzyme used in the feed industry. However, only about 25% to 30% of all industrial enzymes are used for food and feed purposes. It is also worthwhile to note that over 90% of industrial enzymes sales come from less than 30 enzymes, most of them hydrolytic enzymes. As these enzymes are used as an auxiliary material in the food manufacturing process, most of them can be produced using recombinant technology. Thus microbial expression systems play a crucial role for enzyme production.

The market of functional foods more than doubled between 2001 and 2010 and is estimated at 5 billion € (Welck & Ohlig, 2011). Other important biotechnology products are amino acids, vitamins or PUFAs (polyunsaturated fatty acids).

Flavours and fragrances are also biotechnology-relevant markets with a volume of over 22 billion US$ (Leffingwell & Associates, 2011). More than 10% of the supply is derived from bioprocesses, with more than 100 commercial aroma chemicals derived via different biotechnological methods (Berger, 2009). In order to relieve the pressure on natural resources, companies are increasingly turning towards novel biotechnological sources and methods including genetic engineering approaches for the production of these raw materials.

This is also true for a part of the pharma market described below. To supply the anticancer secondary metabolite taxol (paclitaxel) from its original natural source (the bark of the Pacific yew tree Taxus brevifolia) would be impossible. Taxol is produced in minute amounts (0.4 g/kg of bark) and synthetic alternatives including biotechnological steps had to be developed for this blockbuster drug with sales of about 3.5 billion US$. It is difficult to estimate the global market value of botanical or plant derived drugs (BCC Research, 2009), but they seem to grow at a CAGR of 11% and alternative commercially viable expression and production systems will be needed for their production to replace the extraction from endangered or slow growing plants.

### 2.3 The pharmaceutical market

According to the IMS Health Forecasts, the global pharmaceutical market is expected to grow at an overall rate of 5-7% per annum, to reach $880 billion US$ in 2011 (Gatyas, 2011). Of this total pharmaceutical market, monoclonal antibodies alone represented 43 billion US$ and therapeutic proteins 66 billion US$ (2009 figures, Market Research News, 2011). The numbers for small molecule pharmaceuticals, which represent a very fragmented market, are even more difficult to estimate. Franssen et al. estimated the market segment in 2010 for pharmaceutical ingredients using industrial biotechnology at about 20 bn US$ (Franssen et al, 2010). This could be conservative, as the number of small molecule APIs (Active Pharmaceutical Ingredients) using biotechnology in their (chemical) synthesis is rapidly increasing. Blockbuster such as Merck’s Sitagliptin or Pfizer’s Atorvastatin now include biotechnological steps in their manufacturing. Atorvastatin (Lipitor®), a cholesterol-lowering small molecule drug is the largest selling drug in history, which peaked at 12.7 billion US$ annual sales. The chemical synthesis includes a biocatalysis step using a ketoreductase. The same is true for MERCK’s Sitagliptin, the small molecule compound used in the antidiabetes drug Januvia®, which includes a transaminase step in its chemical synthesis. Taking such products into consideration, the number will increase well above 30 billion US$ in 2011. Many classical biotechnological products like antibiotics (market of 42 billion US$ in 2009) or steroids are even not included in these 30 billion US$. What is
important is that the small molecule market remains by far the most important driver for innovation in industrial biotechnology (Meyer et al., 2009).

It is especially red biotechnology with therapeutic proteins and monoclonal antibodies which is thriving and driving growth with established technologies. Remicade was the top selling mAbs brand followed by Avastin®, Rituxan®, Humira® and Herceptin®. The top 5 brands had sales of over $5 billion each. However, besides that they often serve narrower disease phenotypes due to their specificity. The downsides of therapeutic proteins and monoclonal antibodies are that

1. they are not consumable as pills
2. logistics are more complicated due to their instability
3. they are more expensive than small molecules.

Although the market value of small molecule drugs which use one or more biocatalysis steps in their synthesis amounts to well over 30 billion US$ and is growing, the share of biotechnology is well below what it could be. The pharmaceutical industry is under great pressure due to exploding R&D costs, politically due to the health care cost burden and from the market side due to an expected reduction of growth from 7% to 3%.

Both large and small molecules could face a technology gap and manufacturing bottlenecks to meet these production challenges. In both cases we need innovative, sustainable and cost-effective production methods.

The vaccine market is a special subset of the pharmaceutical market, in which innovative solutions are sought for cancer, infectious diseases (e.g. malaria), pandemics (influenza) or bioterrorism. The vaccine market of 20 billion US$/year is expected to grow to 35 billion US$ in five years. Unusual new solutions such as production in transgenic plants may be required to achieve the scale and price targets (Langer, 2011).

The number of market introductions of small molecule pharmaceuticals or NCEs (New Chemical Entities) has been steadily decreasing since the late 1980s, whereas new therapeutic proteins and monoclonal antibodies have increased. To make things worse, small molecule drugs are positioned in markets that are becoming increasingly generic, thereby adding further pressure. The only small molecule drugs with an annual growth of over 10% were high potency drugs and to a lesser extent peptides. However, keeping healthcare costs under control will require efficient and affordable drugs, which are generally smaller entities without complicated and folded structures such as large proteins which need expensive production and logistics and can only be administered by injection.

However, solutions of the manufacturing challenges in the pharmaceutical industry will also provide solutions for other markets.

We need new cost-effective production for large and small molecule drugs (Meyer & Turner, 2009) with more chirality, more complex functionalities, and composed of various chemical structures. Examples are the glycosylation of proteins, aryl- or alkyl-organics drugs for modification of their biological efficacy and the functionalisation of novel biomaterials for medical devices and scaffolds for tissue generation using stem cells. The current biotechnological and chemical toolbox is reaching its technical limits and needs expansion to meet the economic and ecological manufacturing standards of the future (Meyer & Werbitzky, 2011).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Genus, species</th>
<th>Products</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prokaryotes</td>
<td></td>
<td><strong>Small and large molecules</strong></td>
<td><strong>Generalists, well established technology, cost efficient, remains the standard method</strong></td>
</tr>
<tr>
<td>Gram+</td>
<td>Bacillus</td>
<td>Large molecules and enzymes</td>
<td>Host for secreted proteins</td>
</tr>
<tr>
<td></td>
<td>Actinomycetes</td>
<td>Secondary metabolites</td>
<td></td>
</tr>
<tr>
<td>Gram-</td>
<td>Escherichia coli</td>
<td>Small and large molecules</td>
<td>The standard microbial workhorse</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas</td>
<td>Secondary metabolites</td>
<td>Source of biologically active compounds but tricky to manufacture with</td>
</tr>
<tr>
<td></td>
<td>Gluconobacter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myxobacterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eukaryotes</td>
<td></td>
<td><strong>Large and small molecules</strong></td>
<td>Mostly specialist, niche applications with increasing importance</td>
</tr>
<tr>
<td>Fungi</td>
<td>Saccharomyces</td>
<td>Proteins and enzymes</td>
<td>Will lose its importance</td>
</tr>
<tr>
<td></td>
<td>Pichia</td>
<td></td>
<td>Uniform human like N-linked glycans, host for industrial enzymes</td>
</tr>
<tr>
<td></td>
<td>Penicillium</td>
<td>Secondary metabolites</td>
<td>Nutritional flexibility, efficient secreters, industrially widely used</td>
</tr>
<tr>
<td></td>
<td>Aspergillus</td>
<td>Citric acid, enzymes, proteins</td>
<td></td>
</tr>
<tr>
<td>Algae</td>
<td>Chlamidomononas</td>
<td>Mainly small molecules</td>
<td>PUFAs, pigments, in discussion for biofuels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Potential host for human antibodies, expression in 3 genomes</td>
</tr>
<tr>
<td>Plants</td>
<td>Zea mais</td>
<td>Small and large molecules</td>
<td>Cost advantage, vaccines, “plantibodies” (antibodies)</td>
</tr>
<tr>
<td></td>
<td>Nicotiana tabacum</td>
<td>Bovine trypsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pisum sativum</td>
<td>Different products</td>
<td></td>
</tr>
<tr>
<td>Protozoa</td>
<td>Tetrahymena</td>
<td></td>
<td>Expression system for ciliates for the production of therapeutic proteins</td>
</tr>
<tr>
<td></td>
<td>Trochoplusia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insect cells</td>
<td>Spodoptera</td>
<td>Large molecules</td>
<td>Veterinary products, easier cultivation than mammalian cells</td>
</tr>
<tr>
<td></td>
<td>Trochoplusia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>CHO, BHK¹</td>
<td>Large molecules</td>
<td>Several pharma blockbusters, established technology</td>
</tr>
<tr>
<td></td>
<td>CI27, NSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PerC6</td>
<td>Cells, tissues, organs</td>
<td>Mouse cell lines</td>
</tr>
<tr>
<td></td>
<td>Stem cells</td>
<td></td>
<td>Human cell line</td>
</tr>
<tr>
<td></td>
<td>hESC, iPS</td>
<td></td>
<td>Tissue and organ repair, mass production in development</td>
</tr>
</tbody>
</table>

¹ Chinese hamster ovary cells, baby hamster kidney cells

Table 3. This table gives an overview of all biological methods and frequently used genus which can be used for the production of small and large molecules. The microbial pro- and eukaryotes are further tabulated in Table 6.
3. The biotechnology toolbox

Table 3 gives an overview of the production systems available today for the production of small and large molecules. Old technologies still dominate the industry, especially for pharmaceuticals. Other than pharmaceuticals still extracted from plants, animals (mostly now forbidden), only a few biotechnological methods are used. For example of the 130 recombinant protein products in the US and European markets, 48 are expressed in microbes (34% \textit{E. coli} specifically, 1% other bacteria, yeast 13%, see also Figure 6). Another 43% of products are produced in mammalian cells, primarily in CHO cells. \textit{E. coli} and CHO cells have the longest history of use since the commercialisation of the first recombinant proteins in the 1980s. CHO and \textit{E. coli} account for 64% of the expression systems used in manufacturing of currently marketed recombinant therapeutics (US and Europe). Novel expression systems in evaluation for biopharmaceuticals include e.g. \textit{Pseudomonas fluorescens}, \textit{Staphylococcus carnosus}, \textit{Bacillus subtilis}, \textit{Caulobacter crescentus}, \textit{Chrysosporium lucknowense}, \textit{Arxula sp.} as we will see later.

3.1 Cultivation options

While Table 3 lists the organism which can be used, it does not include the cultivation methods which are listed in Table 4 below.

<table>
<thead>
<tr>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free production</td>
</tr>
<tr>
<td>- Single reaction</td>
</tr>
<tr>
<td>- Cascade reactions</td>
</tr>
<tr>
<td>Submersed production of whole cells</td>
</tr>
<tr>
<td>- Suspension culture in sterile reactors</td>
</tr>
<tr>
<td>- Suspension culture in non sterile containments (e.g. open pond raceways)</td>
</tr>
<tr>
<td>- Suspension culture of immobilised and encapsulated organisms</td>
</tr>
<tr>
<td>Solid state (biofilm) production</td>
</tr>
<tr>
<td>- Monoseptic operation</td>
</tr>
<tr>
<td>- Biofilm operation with a mixture of organisms</td>
</tr>
<tr>
<td>Agriculture &amp; Farming</td>
</tr>
<tr>
<td>- Growing of transgenic plants</td>
</tr>
<tr>
<td>- Growing of transgenic animals</td>
</tr>
</tbody>
</table>

Table 4. Overview of the principal biotechnological production methods. Fermentation in sterile containments is by far the most important and suitable for bacteria, fungi, protozoa, algae, plants cells, insect cells, mammalian cells. Stem cells are preferentially produced adherent in biofilms, but the mass production for therapeutic purposes will probably also use suspension culture.

The standard manufacturing procedure in Table 4 is the submersed production of organisms in sterile containments as shown in Figure 1. This method allows the controlled growth of one organisms in a closed tank (fermenter). In over 15 years Armin Fiechter of the Swiss Federal Institute of Technology in Zürich developed a standardised biological test system and systematically tested many different bioreactor designs at the m$^3$ scale. Although many new and interesting bioreactor designs exist, nothing has really changed since the 1980s and the numbers prove that the classic stirred tank with Rushton impellers
remains the most versatile fermenter design (Meyer, 1987). It can be used for unicellular and filamentous cells, and is even used in large scale fermentation for mammalian and plant cell cultures. Moreover, the design gives good performance over a wide range of viscosities. It is a truly multipurpose equipment, with decades of depreciation times for the invested capital for the tanks themselves. The average cost for a large scale sterile fermentation plant (without down stream processing) ranges between 130000 €/m$^3$ for an ISO plant to 300000 €/m$^3$ nominal volume for a cGMP plant for injectable products.

The technology transfer and engineering challenges of fermentation, which include control of the physicochemical environment, mass and gas transfer and other items are discussed elsewhere (Sharma et al., 2011; Meyer & Klein, 2006; Meyer & Rohner, 1995; Meyer & Birch, 1999; Hoeks et al., 1997). Table 4 is a very general overview and many variants of suspension cultures cannot be discussed here. For example the immobilisation of cells by filtration in combination with continuous culture is a powerful tool to reach outstanding productivities with mammalian and microbial cells if sterile operation can be achieved and the cell type allows extended number of cell divisions and generations Hoeks et al., 1992).

Fig. 1. Left side: photograph of a 15m$^3$ state of the art sterile containment for high cell density fermentations. LONZA has built and operates such high performance equipment up to the 75m$^3$ for a range of organisms including methylotrophic yeasts such as Pichia pastoris. The flow breakers are designed as cooling elements needed to remove metabolic heat. The stirrers are concave Rushton impellers which, in combination with specially designed dip pipes (not shown), allow optimal mixing. In most cases the stirrer configuration is completed by the use of a top radially downward pumping marine impeller. Right: Two chromatography columns for the purification of injectable therapeutic proteins with a diameter of 2 meters to demonstrate size and complexity of down stream processing (Copyright@Lonza).

3.2 The production organisms

*The production strain is not everything, but everything is nothing without a good production strain.* Irrespective of the organism used in suspension culture for biotechnological manufacturing, there are numerous common problems, but also some typical differences, which will be discussed. Strain development is, however, the key issue for a commercially viable bioprocess. We have seen above, that companies have invested in expensive multipurpose fermentation equipment. Thus, the biology must be adapted to existing equipment and not
the other way round. Table 5 summarises our experience with regard to the different factors influencing manufacturing costs with mammalian and bacterial cell cultures.

Generally, the strain and its growth characteristics define medium composition, cycle time and final product concentrations. Consequently, the number of steps needed in downstream processing (DSP) and volumetric sterile productivity (the two key cost drivers) are directly related to the choice of strain. The ideal strain is genetically stable, has a high specific \( q_p \) and volumetric productivity \( Q_p \), forms no by-products, and uses a well-defined medium resulting in a DSP with a limited number of steps. Fermentation is where value is created, downstream processing needs to conserve that created value.

A key issue in achieving a high specific and volumetric production rate is the choice of a highly efficient expression system and finally choosing the right recombinant strain for production.

<table>
<thead>
<tr>
<th>Process Flexibility</th>
<th>Effect on ( O_2 )</th>
<th>Effect on sterility</th>
<th>Effect on cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>key</td>
<td>cycle time</td>
<td>key</td>
</tr>
<tr>
<td>Process</td>
<td>limited</td>
<td>limited</td>
<td>medium</td>
</tr>
<tr>
<td>Medium Parameters</td>
<td>limited</td>
<td>limited</td>
<td>usual small</td>
</tr>
<tr>
<td>Plant</td>
<td>very limited</td>
<td>moderate</td>
<td>small</td>
</tr>
<tr>
<td>Fermentation DSP</td>
<td>yes</td>
<td>key product dependent</td>
<td>I&amp;D &amp; Y</td>
</tr>
</tbody>
</table>

Table 5. Effect of strain, process and plant on the overall process outcome. A highly productive strain is the most important factor in a bioprocess. I & D = Interest & depreciation. \( Y = \) Yield.

3.2.1 Prokaryotes and lower eukaryotes

3.2.1.1 Bacteria, yeast and fungi

Prokaryotes such as bacteria and lower eukaryotes such as yeasts or fungi are by far the most productive organisms in biotechnology. We will call them collectively microorganisms in this paper. Because of their small size (Figure 2), microorganisms have by far the largest surface to volume ratio in the living world which allows them to maximize their metabolic rates because of a high rate of exchange of molecules through their surface. With the right cultivation conditions, microorganisms grow exponentially according to the equation

\[ X_t = X_0 \cdot e^{(\mu \cdot t)} \]

\( X_0 \) is the biomass concentration at time zero, or the start of cultivation. \( X_t \) is the biomass concentration at the time of harvest. \( \mu \) is a strain specific growth rate. Some of the fastest growing bacteria weighing maybe \( 10^{-12} \) g are theoretically able to duplicate and grow so fast that their biomass would reach the mass of the earth \( (9 \cdot 10^{24} \text{ tons}) \) in less than a week. This means, that if a bacterial strain produces a protein or another product which can be industrially applied, large amounts can theoretically be produced economically.
Not only are microorganisms able to increase biomass at breathtaking rates, many are also able to grow under different conditions and on a great variety of substrates. This metabolic flexibility requires the ability to produce thousands of different enzymes and other proteins for all sorts of reactions and purposes. With the advent of genetic engineering, these enzymes and proteins can be overproduced in great quantities. Today entire pathways are modified and completed with heterologous genes, which allows the expression and production of molecules foreign to the species.

Fig. 2. Left a budding cell of the yeast *Saccharomyces cerevisiae*. During the reproductive phase a cell multiplies by forming buds. After the buds enlarge, nuclear division occurs and a cross wall is formed between the two cells. *S. cerevisiae* is easy to cultivate at large scale and it serves as host in many biotransformation processes, mainly after genetic recombination. The photograph in the center shows a few cells of the workhorse par excellence in biotechnology: *Escherichia coli*. The diameter of the *E. coli* cells varies between 0.5 and 1.5 micrometer. The bacterium to the right belongs to the genus *Rhizobium*, of which the strain HK1349 is used at large scale for the production of L-carnitine (Copyright@Lonza).

However, it does not end with the ability of microorganisms to produce large amounts of biomass and a great variety of different enzymes and proteins in a short time. There is an even more important reason why one wants to use microorganisms, namely the chemo-, regio- and enantio-selectivity of the microbial toolbox, and this is true for all living matter. No chemical manufacturing technology can match the precision of natural systems for the production of chiral and complex small and large molecules.

While this article focuses on the production of recombinant proteins and small molecules with *Escherichia coli*, it is important to understand the advantages of the different expression and production systems and compare them with the work-horse *Escherichia coli*.

There are now such a multitude of microbial host and expression systems theoretically available (Meyer et al., 2008) that it becomes difficult to choose the right combination. Emerging alternative production systems including those for glycosylated biopharmaceutical proteins for therapeutic use also are numerous (Jostock, 2007). *Escherichia coli* is currently the almost exclusively used prokaryotic production system but alternatives in discussion and developed on top of the commercially available expression systems are listed in Table 6. Examples are *Caulobacter crescentus*, *Proteus mirabilis*, *Pseudomonas* strains, *Staphylococcus carnosus*, *Streptomyces*, as well as fungi and yeasts such as *Pichia*, *Hansenula*, *Arxula*, *Yarrowia*, *Aspergillus* or *Trichoderma*.
<table>
<thead>
<tr>
<th>Name</th>
<th>Microorganism</th>
<th>Commercial Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus megaterium</td>
<td>Bacillus megaterium</td>
<td>Technical University Carolo Wilhelmina</td>
</tr>
<tr>
<td>XS Technologies\textsuperscript{TM}, Bacillus</td>
<td>Bacillus subtilis</td>
<td>Lonza</td>
</tr>
<tr>
<td>Bacillus subtilis (superoxidizing strains)</td>
<td>Bacillus subtilis</td>
<td>University of Groningen Research Corporation</td>
</tr>
<tr>
<td>PurePro Caulobacter expression system</td>
<td>Caulobacter</td>
<td>Wisconsin Alumni Rese Archimod</td>
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<td>Clean genome, stripped down E. coli</td>
<td>Escherichia coli</td>
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<td>Wacker Biotech</td>
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Fig. 3. The fungus *Aspergillus niger* is a “generally recognised as safe” (GRAS) organism used industrially for the production of many substances such as citric acid or gluconic acid, enzymes (e.g. glucose oxidase, glucoamylase, alpha-galactosidase) and other compounds. It is also a versatile host for the heterologous expression of proteins (Copyright@Lonza).

*Aspergilli* (Figure 3) are ideally suited for recombinant protein expression as they have an enormous nutritional flexibility combined with a particularly efficient secretion system and secretion capacity (Fleissner & Dersch, 2010). They are amongst preferred organisms for the production of commercial food enzymes. Genetic engineering of different *Aspergillus* host strains has also allowed the synthesis of industrially relevant amounts of various heterologous proteins (such as human lactoferrin, calf chymosin or the plant-derived sweeteners thaumatin or neoculin peptide sweeteners, Nakajiama et al., 2008). Proteins are also efficiently glycosylated in *Aspergilli*, while undesired hyperglycosylation is usually not observed. Whole genome sequences of several *Aspergillus* species are now available.

### 3.2.1.2 Algae

Recently, *Chlamydomonas reinhardtii*, a unicellular eukaryotic green algae has been proposed as a host to produce several forms of a human IgA antibody directed against herpes simplex virus (Franklin & Mayfield, 2005; Specht et al., 2010). The main reason for turning to algae is the claimed cost advantage and the absence of viral or prion contaminations that can harm humans. One can frequently read that microalgae grow faster (by an order or two of magnitude!) than terrestrial plants and biomass titers of 600-1000 mg/l dry weight are reached. However, one has to be careful. Microalgae can grow very fast when grown heterotrophically on glucose for example. In this case the performance of high cell density fermentations are almost as productive as those reported for bacteria and yeasts (Xiong et al., 2008). But things look different when microalgae are mass-produced phototrophically! For a number of technical and biological reasons not discussed here, doubling time of around 10 hours are probably a realistic assumption, leading to growth rates of $\mu= 0.07$ per hour. This is very low when compared to the bacterium *Escherichia coli* where growth rates of 1 and higher are quite common in large scale. But growth rates are perceptibly higher than with all other eukaryotes except the yeasts and fungi mentioned above. The advantage
over terrestrial plants is the smaller amount of soil used which leads to much better productivities per hectare for algae.

Algae might also have some other distinctive features, which could give them an advantage over other expression system for selected products. For example all three genomes (chloroplast, mitochondrial, nuclear) have been sequenced and can be transformed and each has distinct transcriptional, translational and post-translational properties. Proteins can accumulate at particularly high levels in the chloroplasts because of the absence of the silencing mechanisms. However, proteins in the chloroplasts are not glycosylated. Another feature of algae is, that they can be grown using sunlight or heterotrophically, or using a combination of both.

![Image of Ulkenia sp. algae](link_to_image)

Fig. 4. Polyunsaturated fatty acids (PUFAs) such as docosohexanoic (DHA) acid are important building blocks of human brain tissue or the retina of the eye. The molecule can be produced by fermentation of marine algae. The picture shows the algae *Ulkenia sp.* which is used for the industrial production of DHA. They grow to form “footballs” consisting of single cells – 5 in this case with the product accumulated in the cells as oil drops. The size of the “football” is about 2 μm. Picture by Stefan Geimer, University of Bayreuth, Germany for Lonza (Copyright@Lonza).

The reality is that algae are routinely used for the production of polyunsaturated fatty acids only, and that other small molecule products such as carotenoids (Fernández-Sevilla et al., 2010) are merely in discussion. Although their growth is slow when compared to microbes, algae grow faster than terrestrial plants especially when grown heterotrophically with sugar as carbon and energy source. However, large-scale mass cultivation of algae using sunlight is far from being solved, and the calculations are sobering. Algae using CO$_2$ as a carbon source are a theoretically ideal solution but are a long way from being cost-competitive in practice (Van Beilen, 2010).

Whether algae will ever play a role for recombinant protein production and especially for recombinant monoclonal antibodies is very doubtful for two reasons. Firstly, the industry is very risk averse, conservative and one does not want to change well established production systems. Secondly, there are microbial systems being successfully developed which are also able to provide human-like post translational modifications as we will see later.
3.2.2. Higher eukaryonts

3.2.2.1 Plants

**Whole plants.** Pharming or molecular farming describes the use of transgenic plants (potato, tobacco, banana, tomato, maize, rice, lettuce) and animals for the production of recombinant therapeutic proteins or other recombinant drugs. As with algae, the driver to use plants for recombinant proteins and monoclonal antibodies is the lower unit cost of agricultural production combined with easy scalability while post-translational modifications are possible (Ahmad et al., 2010).

Starting in the late 1980s human interferon, mouse immunoglobulins and human serum albumin were the first recombinant proteins to be tested for transgenic plant production. The first molecular farming project was in 1999 to trigger an immune response in humans with a safe and cost-effective edible vaccine (Langer, 2011). By 2007, about 370 plant made pharmaceuticals (PMPs) were undergoing field trials and about 16 of them were reported to be in clinical trials (Spök, 2007). Recombinant plants are an interesting form of production, and we see important differences to algae.

1. If the product is in an edible form (for example in seeds or fruits) formulation may not be necessary
2. the product in plant material will be easily and stably stored and
3. logistics can be easier as cooling may not be needed for storage and transport.

Because of the cost advantage, it is claimed that insulin could be a candidate for transgenic plant production as diabetes becomes a globally widespread disease, where affordable insulin is an absolute necessity. Vaccines in particular can be interesting products for plant made therapeutic proteins and other pharmaceuticals. MEDICAGO has developed a plant-based manufacturing platform (Proficia vaccine and antibody production system) with PHILIP MORRIS that produces (proficia) vaccine doses (H5N1 flu) in *Nicotiana benthamiana* leaf cells, a wild Australian relative of cultivated tobacco (Vécina et al., 2011). It is a transient expression system based on *Agrobacterium tumefaciens*, infecting plant cells and transferring genetic information to leaf cells. 600 million US$ have been invested in NOVARTIS plant R&D facilities with 167 acres of land. SIGMA ALDRICH uses molecular farming to make avidin, aprotinin, lysozyme, lactoferrin and trypsin at small scale for use as chemicals. TrypZean™ of SIGMA ALDRICH FINE CHEMICALS is a commercial recombinant bovine trypsin from transgenic corn.

Plant seeds are also being investigated as bioreactors for recombinant protein production (Lau & Sun, 2009), because they naturally contain large amounts of proteins but have low protease activities and low water content. Antibodies, vaccine antigens and other recombinant proteins have been shown to accumulate at high levels in seeds and remain stable and functional for years at ambient temperatures. As seeds can be eaten they allow oral delivery of vaccine antigens or pharmaceutical proteins for immunisation where oral delivery is an option. Unfortunately, most protein based therapeutics are usually not biologically active after oral consumption.

However, recombinant production of, for example, proteins in genetically modified plants has also several drawbacks.
1. The time to create a stable transgenic host
2. Low protein titers
3. Extraction and purification from plant organs (if needed)
4. Non mammalian type glycosylation.

Plant transformation using physical methods is rather inefficient (gene gun). Transient expression is less time consuming but limited in scale. Recombinant \textit{Agrobacterium tumefaciens} or plant viruses like the tobacco mosaic virus are used for transient expression.

Let us consider growth rates and use rapeseed as an example as it is one of the industrially used genetically modified crops. At the end of August one hectare is inoculated with 3-4 kg of rapeseeds. At the end of July in the following year (the plant needs a cold period to thrive - the so called vernalisation) the same hectare yields on average 4000 kg of seeds, from which 1700 l of rapeseed oil can be extracted. The biomass has increased by a factor of 1000 in 11 months. This represents a growth rate of $\mu = 0.05 \, h^{-1}$ when calculated for the seeds growing on one hectare. This is relatively high, but it should be considered that one uses 1 hectare during one full year to produce 1'700 l of oil. Using heterotrophic algal fermentation probably 2000 times more oil could probably be produced on the same hectare. But then again, one still needs hectares to produce the necessary carbon and energy source for the fermentation, and we are thus back to square one with algae.

Coming back to transgenic plants and recombinant proteins: because of strong negative pressure from non-governmental environmental organisations and consumer organisations, transgenic plants in general will be slow to be used, also for the manufacturing of therapeutic proteins, unless they offer some of the advantages mentioned, such as edible and cheap recombinant drugs and vaccines for example.

**Plant cell culture.** An alternative to grow whole plants is to grow plant cells in suspension cultures. GREENVAX produces influenza vaccines by growing tobacco using XCELLEREX XDR single use bioreactors. GREENOVATION (Greenovation 2011) proposes bryotechnology (bryophytes = mosses) for recombinant protein production. PROTALIX’s BIOThERAPEUTICS Inc produces the enzyme replacement drug taliglucerase alfa for the treatment of Gauchers disease in disposable 800 litre bioreactors with carrot cells. Despite the fact that large companies such as PFIZER also stake a claim in plant cell-made biopharmaceuticals (taligurase alfa in carrot cells against Gaucher’s disease of PROTALIX, Ratner, 2010), we conclude that commercial heterologous protein production in transgenic plants will not be economically relevant in the foreseeable future.

Whole plants and plant cell suspension culture, both will not play a relevant role in the production of therapeutic proteins and monoclonal antibodies. However, as with whole plants the situation is a different one for the recombinant production of other products and molecules such as secondary metabolites. The shift of wealth from west to east and north to south will fortunately eliminate economic inequalities. But an increasing global prosperity also increases pressure on naturally sourced raw materials especially from plants. Plant cell culture, recombinant or not, is a great alternative and will help to relieve pressure on partly even endangered species.
3.2.2.2 Protozoa

Heterologous expression of proteins or protein fragments is also possible in protozoa. *Dictyostelium discoideum* (Han et al., 2004), *Leishmania tarentolae*, *Perkinsus marinus* and especially *Tetrahymena thermophila* are organisms used, for which recombinant protozoa techniques exist. The ciliate-based expression system (CIPEX) of CILIAN AG (Cilian, 2011) is a proven tool which makes protozoa a potential competitor of mammalian cells such as CHO cells. For example viral influenza haemaglutinin, parasite surface proteins and a human intestinal alkaline phosphatase and a human DNase I were expressed and secreted in the unicellular non-pathogenic protozoan ciliate *Tetrahymena thermophila* (Hartmann et al., 2010; Aldag et al., 2011). The genome of *Tetrahymena thermophila* is entirely sequenced, and it is one of the best-characterized unicellular eukaryotes as it has served for long time as a laboratory model in biology.

Protozoa are naturally mostly feeding on bacteria. However, they can also easily be grown by pinocytosis in a culture medium containing only soluble components. Growth rates of protozoa in non-optimized media and culture conditions reach values of $\mu = 0.02 \text{ h}^{-1}$ and viable cell densities of $1 \times 10^7 \text{ ml}^{-1}$. Protozoa can also be cultivated in normal continuously stirred tank bioreactors and possess the sub-cellular machinery to perform eukaryotic post-translational protein modifications. However, protozoan-based expression systems have not yet made the transition from a laboratory model to an established recombinant protein platform at large scale. The main advantage of protozoa is that they are free of endogenous infectious agents as their genetics and phylogenetic distance to higher animals make viral infection unlikely. As with other expression systems high gene doses allow relatively high volumetric productivities $[Q_p]$ and scale-up has been proven up to the 1.5 m$^3$ scale. Protozoa have a consistent oligo-mannose N-glycosylation albeit not of mammalian nature.

![Fig. 5. Picture of *Tetrahymena thermophila*, one of the best characterized unicellular non-pathogenic protozoa, expressing a recombinant green fluorescent protein in phagosomes; protozoa can also secrete recombinant products. The ciliates, distributed on the cell surface, cannot be seen because the photograph was taken with a light microscope. The cell measures about 50 $\mu$m in diameter (Copyright@Cilian).](image)

In the case of *Tetrahymena thermophila* (Figure 5) and with optimized media and culture conditions, cell densities of over $2 \times 10^7$ cells per milliliter and dry biomass titers of 8 g/l can
be reached in high cell density protozoa fermentations and 50 g/l in continuous fermentations with cell retention. Generation times become very short and values between 1.4 and 3 hours can be obtained.

In conclusion, protozoa such as *Tetrahymena thermophila* have a certain potential for the recombinant production of selected recombinant proteins. To some extent they combine the advantages of the microbial and the mammalian world as they can grow rapidly to relatively high cell densities and have a posttranslational modification apparatus. If the necessary tools for the manufacturing with stable expression, conserved sequences and target glycosylation can be established, we may see cultivation of protozoa in large scale fermenters in the future for human enzymes, monoclonal antibodies and, in particular protein vaccines. Because of their faster growth, the first vaccine targets in preclinical phase are already in development using protozoan recombinant expression.

3.2.2.3 Insect cells

**Insect cells culture.** A few recombinant proteins made using insect cell lines have already been approved for veterinary use. Only one vaccine, Cervarix® of GLAXO SMITH KLINE, has been approved for human use within the EU (in 2007) and in the US in 2009. Production with insect cells has the following advantages:

1. easy to culture and faster than many mammalian cell lines
2. high tolerance of osmolality
3. advantageous and low ratio by-product vs expressed product

The *Spodoptera frugiperda* cell lines (Sf-9 and Sf-21, Figure 6) and *Trichoplusia ni* are frequently used host cell lines for recombinant protein expression and production via infection with a genetically modified baculovirus expression vector system, BEVS (Invitrogen, 2011; Quiagen, 2011; Tiwary et al., 2010). As for other cell lines used for production, insect cell lines need to be “immortalized” or rendered permanent.

![Image of moth](https://example.com/moth.jpg)

Fig. 6. Cells from the ovarium of this moth *Spodopera frugiperda* are used in combination with baculoviruses for the recombinant production of proteins in suspension culture in fermenters. The method was first introduced in 1982 and in 1985 the first recombinant protein interleukin-2 was expressed in moth cells (Copyright@Canadian Biodiversity Information Facility, 2011).
Taticek et al. (2001) compared the growth and recombinant protein expression in suspension culture and attached cells culture of *Spodoptera frugiperda* and *Trichoplusia ni* (Taticek et al., 2001) expressing *Escherichia coli* beta-galactosidase or human secreted alkaline phosphatase. The production of both enzymes varied as a function of inoculum size, media, culture conditions etc. Cell densities of $5 \times 10^6$ viable cells/ml and doubling of 20 hours were reached, which corresponds to a growth rate of about $\mu = 0.04 \, \text{h}^{-1}$. It is not clear whether a typical large scale fed batch cycle for insect cells would also be around 4 to 5 days which would about 2 to 3 times faster than a batch with mammalian cell culture. However, generally insect cells have two major disadvantages:

1. the baculovirus system results in cell death and lysis of the host insect cells and the release of cell proteins, which offsets again the high productivity of the cells
2. N-glycosylation of insect cells is different from mammalian cells.

Vermasvuori et al. compared the production of a model protein (Negative factor or Nef) in *Escherichia coli*, *Pichia pastoris* and the *Drosophila* S2 cell line (Vermasvuori et al., 2009). When studying the systems purely economically the microbial system had significantly lower manufacturing costs than the insect cell lines. The most significant difference between the manufacturing costs of the two microbial systems was due to the much longer strain construction time with the *Pichia pastoris*. The manufacturing costs for the production of 100 mg Nef protein were

1. *Escherichia coli* 6456 €
2. *Pichia pastoris* 13382 €
3. *Drosophila* S2 21111 €

Omitting the strain construction costs, the microbial systems were cost-wise fairly comparable. These numbers from small scale experiments insinuate that insect cells could be theoretically a threat for mammalian cell culture. However, there are no compelling data showing an advantage by switching to insect cells for large scale production.

**Transgenic insects.** The production of recombinant proteins in live animals is an option, as production of therapeutic proteins and monoclonal antibodies is an option in living plants. The most frequently proposed method is that of producing and extracting recombinant proteins from the milk of transgenic livestock. However, insects such as silkworms (Fraser & Jarvis, 2010), can theoretically also be used for recombinant protein production. However, we believe that transgenic insects will not play a role in the production of pharmaceuticals rather than in the control of insect populations and prevention and control of parasites and disease transmission in man, animals and plants.

**3.2.2.4 Mammalian cells**

**Mammalian cells in culture.** The clinical and commercial success of mainly monoclonal antibodies has led to the rapid development of mammalian cell culture within a short time after the landmark findings of Cesar Milstein and Georges Köhler in the mid 70s. Mammalian cell culture is a well established production method for the production of therapeutic proteins and monoclonal antibodies. One of the most frequent mammalian hosts used is the CHO cell line (Chinese hamster ovary) in combination with the GS expression system or the DHFR (dihydrofolate reductase) expression system (Birch & Racher, 2006). The mouse NS0 cells cell line is another option which is less frequently chosen today.
Continuously stirred tank fermenters are operated (Varley & Birch, 1999) with volumes of up to 20 m$^3$. An average batch lasts about two weeks with specific productivities up to 100 pg/cell x day. Product titers for antibodies of several grams per litre are almost routine today, making product recovery and purification the greater challenge from a manufacturing perspective. Defined media are available for high growth rates, stable expression, low lactate and byproduct formation to facilitate fermentation, isolation and purification. Lonza (Lonza, 2011) recently introduced a novel medium and feeding platform for its GS-expression system$^\text{TM}$, which leads to product titers of up to 10 g per litre!

Rapid and efficient development of stable production cell lines is a critical step and transfection alternatives for the quick preparation of stable mammalian production cell lines for recombinant proteins are needed (Wurm, 2004; Birch et al., 2005). The PiggyBac transposon is an example of a novel delivery vehicle for rapid and efficient recombinant cell line generation (Matasci et al., 2011).

Unfortunately, the generation of a stable production cell line remains a time consuming endeavour. However, for early feasibility tests with a protein, transient gene expression and protein production may be sufficient (Ye et al., 2009). EXCELLGENE SA (Excellgene, 2011) has successfully established transient gene expression for fast protein delivery. Production and recovery of several grams of purified protein can be done within 3-4 weeks. However, transient recombination is limited by two factors:

1. the need for large amounts of plasmid DNA for transfection
2. the requirement for cell culture medium exchange before transfection

Nevertheless, transient technology allows a reduction of cost and time for biopharmaceuticals with mammalian cells. It is not yet a valid method for regular production or even material for clinical material testing of e.g. drug candidates. Cell lines may not be stable but at least production can start within a matter of weeks. The combination of transient expression with disposable reactors, which are now available up to the m$^3$ scale (Eibl et al., 2010), makes the production of several 100 g of new proteins possible in a short time.

**Transgenic animals.** Clones and transgenics of livestock and companion animals have become a reality in animal farming, meat production and even pharmaceutical production since the first transgenic animals were created in the laboratory. Mice were the first mammals to be genetically modified in the early 1980s and in 1987 the breeding of a tPA (human tissue plasminogen activator) producing transgenic mouse was reported by Simons et al. (1987). As with any other organism mentioned above, it is also possible to introduce or delete existing genes in an animal cell and have the modification passed on to the next generation resulting in a new phenotype of the living animal.

In 2004 about a dozen companies had products in development in transgenic animals (Keefer, 2004) but many of them have been abandoned. Human antithrombin III (hAT) of GTC BIOTHERAPEUTICS, a glycoprotein controlling blood clotting, was the first approved biopharmaceutical from transgenic animals. NEXIA BIOPHARMACEUTICALS published a patent in 2002 for the production of spider silk biofilaments in transgenic animals using milk or urine-specific promoters (Karazas & Turcotte, 2003) but the process is not competitive with microbial production. The Dutch company GEN PHARMING was a pioneer in the field of production of human lactoferrin in ruminants. Alpha 1-antitrypsin, fibrinogen,
tissue plasminogen activator, vaccines, human monoclonal and polyclonal antibodies are other example of products.

This technology has, however, been the subject of controversial discussions (for example “cloned meat”) as these biotechnology applications have been judged with evident hierarchies of acceptability (Einsiedel, 2005). Transgenic animals are particularly sensitive but they nevertheless offer options to produce a therapeutic protein in a fluid of the animal (milk, blood, urine) of mice, goats or cows. Coupling the target protein to a signal one can direct the expression into the mammary glands and milk - the optimal choice.

Nonmammalian animals such as birds or insects (see above) can also be used for the production of glycosylated therapeutic proteins and monoclonal antibodies, especially in the egg white or egg yolk of transgenic chicken.

Patel et al., 2007 claim, that animals are more cost effective bioreactors, with 16 therapeutic proteins in development in transgenic animals (sheep 5, pig 2, goat 4, cow 5) plus several monoclonal antibodies in cows, goats and chickens. Productivities in terms of milk containing recombinant protein given are ~8000 litres containing a total 40 – 80 kg of recombinant protein. However we disagree, and think that transgenic animals will only be used in a very few and exceptional cases for the following reasons:

1. low success rate of gene transfer (e.g. 0.1% with cows)
2. cost and time to produce transgenic animals
3. possible infectious agents from mammals
4. low consumer acceptance in view of better alternatives
5. many alternatives exist

Take productivity as one example, a single cow will produce ~60 kg of a therapeutic protein in one full year which has still to be isolated and purified. In the case of a goat or a sheep that number drops to 4 kg and 2.5 kg per year, respectively. This should be compared with the productivity of a 20m$^3$ bioreactor with mammalian cells which produces on average 125 kg in a single month! That means one bioreactor replaces 25 cows, 375 goats or 600 sheep.

How do depreciations between 25 cows and a 20m$^3$ plant differ? The cost of one transgenic animal is high, 500000 US$ for one calf according to Keefee (2004). This is due to inefficiencies in this technique as over 1000 bovine, 300 sheep and 200 goat oocytes must be injected. Goats and sheep with their shorter generation interval are less costly, and the figures may look somewhat better today. On the other hand and based on numbers given by Patel et al (2007), we calculated an average of about 50000 US$ in value created per animal and per year. It now all depends how fast a productive herd can be created by cloning and how long the productive period of a cloned transgenic animal is? Over how many productive years do we have to depreciate the animals? One important disadvantage of a capital investment in a transgenic cow is that a cow is a dedicated “plant” while a bioreactor is a multipurpose and multiproduct installation.

We do not believe that transgenic animals are an attractive alternative except for a few very special exceptions. However, the one argument for transgenic animals (as for transgenic plants and plant seeds) is the case in which the product can be consumed directly with the milk. It would make therapy affordable, and one can imagine that an oral protein-based vaccine would be an ideal candidate for a transgenic animal.
Fig. 7. Microbial and mammalian cell culture are used in 93% of all cases for the production of therapeutic proteins. See also Figure 8 with the spread of the individual expression systems.

In summary, microbial fermentation and mammalian cell culture will continue to carry the main burden for the production of recombinant proteins as it is already the case today (Figure 7). Other expression systems, especially plant-based and algae, will have potential for recombinant protein niche applications. The situation is different for small molecule pharmaceuticals, neutraceuticals and fine chemicals, where a more varied host-expression system combination will be needed. However, even in the latter case one will first fall back on proven methods. We will now describe in more details the beacon in recombinant microbial expression – *Escherichia coli*.

4. *Escherichia coli* as work horse

In the year 1885 the German paediatrician Theodor Escherich (1857-1911) described a bacterium, which he called “*Bacterium coli comunale*”. At that time nobody could anticipate that this bacterium, which later on was named after him *Escherichia coli*, would become world famous as a model organism in the field of molecular biology and as “the” minifactory for recombinant protein manufacturing (Piechocki, 1989).

This is best demonstrated by statistical figures related to expression platforms in use (Figure 8). In the reported year 34% of all recombinant therapeutic proteins registered in the US and EU were produced by means of *Escherichia coli* based expression technology. The second and third most successful expression platforms were Chinese Hamster Ovary cells with a 30% and yeast systems, mostly *Sacharomyces cerevisiae*, with a 12% shares respectively (Rader, 2008).

4.1 Why is *Escherichia coli* such a popular expression host?

Although there is no gold standard platform in microbial expression, expression systems based on *Escherichia coli* have dominated microbial expression for more than 30 years. One can only speculate on the reasons for this domination. *Escherichia coli* and its phages were early objects and models for studying molecular biology topics, especially aspects related to the understanding of gene functions and regulation. More than 10 scientists received the Nobel prize for exciting discoveries connected to research on *Escherichia coli* (Piechocki, 1989). Worth mentioning is the isolation and purification of a restriction enzyme for the first time by Werner Arber in 1968. These enzymes are enabling tools in the area of rDNA technology. The rapid pace in the development of expression technology and of genetic
engineering tools is best reflected by the quite early launch of a first biopharma product, expressed in *Escherichia coli*, recombinant human insulin in 1982 (Humulin®, licensed by GENENTECH to ELI LILLY). This is even more remarkable if one considers the lengthy approval procedure for therapeutics. *Escherichia coli* based biotechnology profited directly from the multitude of fundamental discoveries made on this model organism, giving this species a timely technical advantage in use as expression host.

![Bar chart showing percentage of expression platforms used](image_url)

**Fig. 8.** Percentage of expression platforms used for the manufacture of bio-therapeutics in the US and the EU. The figure is based on numbers published by Rader (2008).

Other explanations for the success of this microorganism are low genome complexity and the extra-chromosomal genetic elements, plasmids, which ease both (a) in-vitro manipulation of genetic elements and (b) insertion of homologous and foreign genes into the organism.

Besides low safety concerns and high regulatory acceptance, ease of use and familiarity with the organism was in favour of *Escherichia coli*. There is hardly a student in biology who has not run at least one cloning experiment in one of the *Escherichia coli* expression systems used in academia. Since its first industrial applications, *Escherichia coli* expression technology has been continuously improved with the aims of gaining control of the quality of the recombinant products and increasing the product titre in fermentation, the latter obviously being crucial to process economy.

**4.2 What are the characteristics of an industrial *Escherichia coli* expression platform?**

Incremental improvements led to the development of *Escherichia coli* based expression platforms that are suitable for industrial use. More precisely these systems allow for robust, reliable and scalable processes and economical manufacturing. High performance expression technology is characterized by two properties: (a) high volumetric productivity $Q_p$, preferentially due to a high specific product production rate $q_p$, and (b) high control on product quality, meaning that no or only a negligible amount of product variants are produced.
Industrial expression systems distinguish themselves from academic systems by an optimized combination of the various components of which an expression system is made. Basically, a bacterial expression system is composed of a host and a vector which contains the product coding DNA, a selection marker and various regulatory elements. Regulatory elements are promoters, signal sequences, ribosome binding sites, transcription terminators and vector replication or integration regions.

**Host.** The host organism provides specific features to an expression system as a result of its genetic background; these features include:

1. growth characteristics such as specific growth rate \( \mu \)
2. maximum achievable cell densities
3. nutritional needs
4. robustness at cellular and genetic level
5. control of product degradation
6. secretion capacity preferentially into the medium
7. amount of endotoxins produced
8. post-translational modifications

High cell densities are most desirable since a positive correlation exists between the amount of biomass (\( X \)) and the product production rate (\( r_p \)). The corresponding equation is

\[ r_p = q_p \times X \]  

(product production rate = specific product production rate \( q_p \) x biomass).

The relationship above should not be confused with growth rate dependency on the product production rate, which can be optimal at high or low growth rates. It is possible that in the worst case maximal specific production rates \( r_p \) correlate with very low growth rate close to maintenance (Meyer & Fiechter, 1985). In that case production requires two separate phases, growth and production phase.

Commonly used *Escherichia coli* host strains are listed in Table 7. BL21 is the most frequently used *Escherichia coli host*. BL21 popularity is based on

1. *lon* and *ompT* protease deficiencies
2. beneficial growth and metabolic characteristics
3. insensitivity to high glucose concentration.

The organism is not sensitive to high glucose concentration due to its active glyoxylate shunt, gluconeogenesis and anaplerotic pathways and a more active TCA cycle, which leads to better glucose utilisation and lower acetate production (Phue et al., 2008). However, when used in combination with the T7 expression system and when exposed to stress, this host is at risk of bacteriophage DE3 excision. For this reason laboratories started to promote the use of BLR, a recA\(^{-}\) mutant of BL21. In our experience an increased use of W3110 is taking place in the industry. This can be attributed to the excellent production capabilities of this host. Orgami strains may allow for better formation of disulfide bonds in the cytoplasm due to lower reducing power in the cytoplasm (Novagen, 2011). The endA\(^{-}\) and recA\(^{-}\) hosts DH5\(\alpha\) and JM109 are the organisms of choice for the manufacture of pDNA. The lack of endonuclease 1 which degrades double stranded DNA positively affects stability of pDNA (Phue et al., 2008). In conclusion, product nature and product characteristics determine the selection of the most optimal host.
**Escherichia coli Host Strains**

<table>
<thead>
<tr>
<th>Strain Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21</td>
</tr>
<tr>
<td>K-12 strain, recA^-</td>
</tr>
<tr>
<td>HMS174</td>
</tr>
<tr>
<td>K-12 strain, recA^- mutant of BL21 with decreased likelihood of excision of DE3</td>
</tr>
<tr>
<td>BLR</td>
</tr>
<tr>
<td>K-12 or B strains with mutations in trxB and gor</td>
</tr>
<tr>
<td>Orgami strains</td>
</tr>
<tr>
<td>K-12 or B strains which supply tRNAs for codons that are rare in E. coli</td>
</tr>
<tr>
<td>Rosetta strains</td>
</tr>
<tr>
<td>K-12 strain</td>
</tr>
<tr>
<td>W3110</td>
</tr>
<tr>
<td>K-12 strain</td>
</tr>
<tr>
<td>MG1655</td>
</tr>
<tr>
<td>K-12 strain</td>
</tr>
<tr>
<td>RV308</td>
</tr>
<tr>
<td>K-12 strain</td>
</tr>
<tr>
<td>DH5α</td>
</tr>
<tr>
<td>K-12 strain, recA^-, endA^-, often used for pDNA manufacture</td>
</tr>
</tbody>
</table>

Table 7. Frequently used *Escherichia coli* host strains and related specific characteristics.

**Promoters.** Promoters control the expression to the extent of how much and at which point in time mRNA is synthesized. As a consequence they control production of product. A large number of promoters that allow modulation of the mode of induction in a desired way are used in the industry. Lactose or lactose-analogue IPTG induced T7 promoter-based expression systems currently dominate the market. Apart from T5, araB and phoA, other classical promoters such as lambda, lac, trp, P_L, P_R, tetA and trc/tac are rather seldom used.

Novel promoters are under development and continuously make their way into industrial applications. New disaccharide inducible promoters, which induce protein production during the stationary growth phase, have recently been successfully applied in *Escherichia coli* based biopharmaceutical processes. Some of these are part of Lonza’s XS Technologies™ *Escherichia coli* platform, which has been chosen as an example to discuss performance of current leading industrial *Escherichia coli* expression platforms (Lonza). Depending on the promoter the induction signal is of a chemical or physical nature. Some of the above mentioned *Escherichia coli* promoters have been successfully used in other bacterial systems such as *Bacillus subtilis* (Alexander et al., 2007).

State of the art industrial expression platforms allow for product specific modulation of the rate of protein synthesis. Proteins of high complexity, having disulphide bonds are typically best produced at a lower production rate. In contrast proteins of low complexity are often produced at a high production rate, thus achieving high concentrations after a short time of fermentation. Productivity is often affected by interaction between specific promoters and recombinant target proteins. Therefore, in general, it makes sense to screen for the performance of different promoters.

**Signal Sequences.** Signal sequences determine whether a product is directed through the cellular membrane and out of the cytoplasm; the signal sequence is cleaved during the secretion step. Secretion is desirable in many cases, since a large proportion of target proteins do not fold correctly in the reducing cytoplasmic environment. Folding requires oxidative conditions which are provided outside the cytoplasm. Secretion sequences
frequently used in *Escherichia coli* are MalE, OmpA and PelB. Yeast organisms such as *Saccharomyces, Pichia, Hansenula, Yarrowia* and Gram-positive bacteria such as *Bacillus* and *Corynebacterium* secrete proteins which carry a secretion signal into the medium, whereas Gram-negative genera such as *Escherichia, Pseudomonas* and *Ralstonia* direct the product through the inner membrane into the periplasmic space. This is what the theory says. According to the authors’ experience, the *Escherichia coli* outer membrane is leaky for a large proportion of secreted proteins which are supposed to accumulate in the periplasmic space. The observed partitioning of the secreted protein between fermentation medium and periplasmic space can be influenced to some extent by modifying the fermentation conditions. The latter behaviour is product dependent and for the time being not predictable.

**Selection markers.** Selection markers are necessary for the cloning process and crucial for controlling plasmid stability. Typical microbial selection markers are antibiotic resistance genes. However, the prevalence of β-lactam allergies strongly suggests avoidance of the use of ampicillin and other β-lactam derivatives for the purpose of selective pressure in the manufacture of clinical products. Optional stabilization systems used in *Escherichia coli* are based on antidote and poison gene systems with the poison gene being integrated into the bacterial chromosome and the antidote gene located on the plasmid, respectively (Peubez et al., 2010). Constitutive expression of the antidote gene stabilizes plasmid-containing cells. A system based on the mode of action described above is marketed by DELPHI GENETICS Inc (Delphigenetics, 2011).

Besides the above mentioned regulatory aspect, Rozkov et al. (2004) note another one that should be taken into consideration when selecting the plasmid stabilizing system. According to these authors, the presence of an antibiotic selection marker imposes a huge metabolic burden on an expression system. They found that the product of the selection marker gene accounted for up to 18% of the cell protein. A negative effect on the recombinant expression of the genes of interest is highly likely. Due to constitutive expression this is the case even in the absence of the corresponding antibiotic in the medium. One way to circumvent this problem is to use complementation markers, i.e. marker genes that complement an auxotrophic chromosomal mutation.

A majority of successful technologies, genetic elements and related know-how, are subject to patent protection or trade secrets, as shown also in Table 6. In particular, multiple license requirements for the use of a specific production technology can lead to an unfavourable economic situation. On the other hand, off-patent expression systems and elements thereof are usually not state of the art. Since process economy depends to a large extent on productive and robust strains, outsourcing strain development to a specialised laboratory is often justified, given that licensing cost remain reasonable. The resulting economic benefits on the process side typically offset the costs related to accessing a productive and robust state of the art industrial strain platform.

**4.3 A more critical view on *Escherichia coli* expression platforms**

Despite their dominant position within microbial expression *Escherichia coli* based expression platforms also exhibit weaknesses which should not be ignored. These drawbacks are shared with other commercialised Gram-negative expression platforms as *Pseudomonas* and *Ralstonia*. Among these disadvantages are
1. the presence of high levels of endotoxins that need to be removed from therapeutic products
2. the difficulty of controlling full secretion into the medium.

WACKER Chemie has commercialised a K-12 derivative that exhibits higher secretion ability than other K-12 and B strains (Mücke et al., 2009). Other expression system aspects such as:

1. the lack of posttranslational modification capability including a lack of glycosylation machinery
2. the capability of intracellular expression
3. the difficulty of expressing complex, multimeric proteins with a high number of disulfide bonds

are often referred to as disadvantages. These apparent drawbacks can, however, be turned to advantages depending on the target protein’s specifics.

Table 8 compares the suitability of the 3 leading expression platforms related to characteristics of the expression candidate protein. Apart from the two characteristics (a) requirement for human-like glycosylation, which includes monoclonal antibodies whose efficacy depends on Fc effector functions and (b) peptide nature of the recombinant target, most of the aspects captured in the table, do not give a clear indication regarding choice of the ideal expression platform. There is a large grey zone which typically needs to be explored empirically.

Active enzymes up to a size of 220 kDa and 250 kDa recombinant spider silk protein have been successfully expressed in *Escherichia coli* at high concentrations, questioning the dogma that bacterial systems are not suitable for the expression of large proteins. This thesis is further supported by successful expression of complex heterodimers, such as aglycosylated functional antibodies, in bacterial systems. For an in-depth analysis of expression of complex heterodimers in *Escherichia coli* we recommend the paper of Jeong et al. (2011). We also question the criticism towards inclusion body formation that often is cited as a disadvantage. Rather than a drawback we consider this as a capability that adds flexibility to the use of *Escherichia coli* based platforms. Industrial expression platforms allow for inclusion body concentrations as high as 10 g/l culture broth and above. This consideration combined with an efficient refolding process provides high potential for a competitive process from a cost point of view.

Some therapeutic protein candidates are not glycosylated, such as a non-glycosylated version of an antibody. In particular, recombinant proteins produced by yeast expression systems may carry undesired O-glycans. In these cases a lack of glycosylation capability can be considered as advantage rather than a system weakness. Intracellular expression in *Escherichia coli* may lead to product variants (a) with N-terminal formyl-methionine and (b) without formyl-methionine at the N-terminus. Methionine cleavage by the methionyl-aminopeptidase depends on the characteristics of the adjacent amino acid, which consequently determines the ratio of the 2 product fractions.

Earlier on endotoxin formation and low control of secretion into the medium were mentioned as problematic aspects for expression systems which are based on Gram-negative bacteria such as *Escherichia*, *Pseudomonas* and *Ralstonia*. On the other hand Table 8 also
indicates some weaknesses of yeast platforms. On the one hand yeast N- and O-glycosylation capability can negatively impact product quality so that adverse immunogenic reactions in the clinic are the result. Another problem often observed with *Pichia* and *Hansenula* are product variants produced through incomplete N-terminal processing and proteolytic degradation (Meyer et al., 2008). This, together with an on average lower observed productivity, negatively affects broad usage of yeast systems, despite their advantageous secretion capability.

<table>
<thead>
<tr>
<th>Protein Characteristics</th>
<th>Bacterial (Gram-) Systems</th>
<th>Yeast Systems</th>
<th>Mammalian Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>size: small to mid size</td>
<td>• • •</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>size: large proteins</td>
<td>• • 1)</td>
<td>• •</td>
<td>••</td>
</tr>
<tr>
<td>peptides</td>
<td>• • • 2)</td>
<td>• • •</td>
<td>•</td>
</tr>
<tr>
<td>monomers</td>
<td>• •</td>
<td>• •</td>
<td>•</td>
</tr>
<tr>
<td>homo-multimers</td>
<td>• •</td>
<td>• •</td>
<td>•</td>
</tr>
<tr>
<td>hetero-multimers</td>
<td>•</td>
<td>• •</td>
<td>•</td>
</tr>
<tr>
<td>disulphide bonds (folding)</td>
<td>• 3)</td>
<td>• • •</td>
<td>•</td>
</tr>
<tr>
<td>hydrophilic proteins (soluble)</td>
<td>• • •</td>
<td>• • •</td>
<td>•</td>
</tr>
<tr>
<td>hydrophobic proteins (low solubility)</td>
<td>• • 4)</td>
<td>• • •</td>
<td>•</td>
</tr>
<tr>
<td>human (like) glycosylated</td>
<td>-</td>
<td>• 5)</td>
<td>• • •</td>
</tr>
<tr>
<td>not-glycosylated</td>
<td>• • •</td>
<td>• • •</td>
<td>•</td>
</tr>
<tr>
<td>Protein prone to proteolytic digest</td>
<td>• • •</td>
<td>• 6)</td>
<td>• • •</td>
</tr>
<tr>
<td>(N-terminal product variants)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Criteria that drive selection of an expression platform. Legend: -, not suitable; • low, •• medium, ••• high suitability; 1) mostly cited as limiting criterion, nevertheless, up to 220 kDa proteins have been expressed in Lonza’s *E. coli* XS Technologies™ platform with a very high titre, 2) Unigene and Lonza developed *E. coli* based peptide platforms, 3) secretion required for most recombinant proteins, 4) proteins exhibiting low solubility or a high aggregation propensity are often expressed at high titres as inclusion bodies, 5) yeast type glycosylation, mainly mannose comprising oligosaccharides, is highly immunogenic, 6) N-terminal product variants are frequently observed with *Pichia pastoris* and *Hansenula polymorpha* as a result of incomplete N-terminal processing.

Table 9 compares bacterial Gram-negative and yeast platforms to selected bacterial Gram-positive expression platforms, i.e. to *Bacillus* and *Corynebacterium* platforms (White, 2011). The comparison suggests that the disadvantages of the existing bacterial Gram-negative platforms and yeast platforms can be overcome by moving into bacterial Gram-positive platforms. Gram-positive bacteria, in contrast to Gram-negative bacteria do not produce endotoxins and they naturally secrete proteins. Comparing them to yeast, they do not
glycosylate proteins and there are no N-terminal processing problems. Both *Bacillus* and *Corynebacterium* hosts need to be engineered to resolve the problematic aspects of the corresponding wildtype strains such as low plasmid stability and secretion of undesired proteases.

<table>
<thead>
<tr>
<th>Problematic Characteristics</th>
<th>Yeast Platforms</th>
<th>Gram+ Platforms</th>
<th>Gram- Platforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Pichia</em> Hansenula</td>
<td><em>Bacillus</em> Corynebacterium</td>
<td><em>Escherichia</em> <em>Pseudomonas</em></td>
</tr>
<tr>
<td>Endotoxins</td>
<td>suitable</td>
<td>suitable</td>
<td>not suitable</td>
</tr>
<tr>
<td>Control of secretion</td>
<td>suitable</td>
<td>suitable</td>
<td>not suitable</td>
</tr>
<tr>
<td>N-terminal product variants</td>
<td>not suitable</td>
<td>suitable</td>
<td>suitable</td>
</tr>
<tr>
<td>Undesired glycosylation</td>
<td>not suitable</td>
<td>suitable</td>
<td>suitable</td>
</tr>
</tbody>
</table>

Table 9. Suitability of yeast, Gram-negative and Gram-positive expression platforms related to classical microbial platform weaknesses.

### 4.4 Production performance of relevant industrial *Escherichia coli* expression platforms

In contrast to the expression of antibodies in CHO cells, expression success cannot be predicted in microbial expression systems. What is good for a specific recombinant protein A does not necessarily work for protein B, even if B is a protein variant of A. An integral part of the various strain platforms are generic high cell density fermentations. When considering industrialisation, strains and fermentation procedures should be looked at as single entities rather than separate process aspects. This is the main reason for the difficulty in judging the performance of expression platforms in general. Data from one single product are not sufficient, since the performance of one expression platform can differ greatly from product to product for as yet unknown reasons. One platform typically shows exceptional productivity only for a small number of products and rather low productivity for the majority of desired expression targets.

Expression titres of commercial products are typically handled as trade secrets. The authors have access to an informative set of expression titre data of leading *Escherichia coli* expression systems which are part of Lonza’s XS Technologies™ platform (Figure 9). This platform is a broad one which in itself encompasses various *Escherichia coli*, *Pichia pastoris* and *Bacillus subtilis* platforms. In our experience, heterogeneity of the recombinant protein pipeline demands access to a variety of powerful expression tools in order to cope with specific expression challenges. On a few occasions the platform performance could be directly benchmarked against competitive CMO and other commercialised platforms based on *Escherichia coli* and *Pseudomonas*. On these occasions XS Technologies™ showed superior or equal performance. Therefore we consider the performance data shown in Figure 10 as representative for leading bacterial Gram-negative expression platforms.
Fig. 9. Example of an industrial expression platform, XS Technologies™ (Lonza). The platform comprises a number of powerful expression technologies for expressing recombinant proteins in *Escherichia*, *Pichia* and *Bacillus* in order to cope with the expression challenges related to the heterogeneity of the recombinant proteins pipeline, including recombinant peptides and pDNA.

With Gram-negative organisms such as *Escherichia coli* and *Pseudomonas*, the recombinant product can be localized in different spaces, either intracellular (cytoplasmic) or extracellular. We define the latter as proteins expressed with a secretion sequence, and thus directed through the inner membrane, which means that the recombinant protein can be localized either in the periplasm or in the cell free medium. As a second aspect to consider, product is formed in either a soluble form or as insoluble aggregates. Apart from intentional inclusion body formation, production in a soluble, functional form is preferred. Therefore 4 effective expression modes are to be distinguished. Recombinant protein can be localised (C1) in the cytoplasm, insoluble as inclusion bodies, (C2) in the cytoplasm in a soluble form, (C3) in the cell-free medium in a soluble form and (C4) in the periplasm in a soluble form. Periplasmic insoluble material is typically not accessed and therefore ignored in the productivity figures.

Figure 10 shows expression levels of 24 recombinant proteins, mostly biopharmaceuticals that are expressed in *Escherichia coli* platforms. Induction is platform-dependent either by the addition of a corresponding sugar or by entering the stationary phase.
Fig. 10. Expression titres obtained for 24 different recombinant proteins mostly biopharmaceuticals. The proteins were expressed in either one of the sugar inducible or one of the stationary phase inducible *Escherichia coli* systems belonging to Lonza XS Technologies\textsuperscript{TM} platform. Among the 24 recombinant products were fragment antibodies, Fab-fusions, single-chain antibodies, virus-like particles, novel non-antibody type binders, growth factors, recombinant enzymes, amphipathic proteins, recombinant vaccines, peptides (hormones and others), affinity ligands and monomers of biopolymers; size of the proteins varied between 2 and 220 kDa. Legend: C1, insoluble as inclusion bodies in cytoplasm; C2, soluble in cytoplasm; C3, soluble in cell-free medium; C4, soluble in periplasm.

**Cytoplasmic expression (categories C1 and C2).** Among the products expressed in the cytoplasm, either soluble or insoluble as inclusion bodies, were highly soluble recombinant proteins as well as proteins prone to high aggregation propensity belonging to product classes such as recombinant vaccines, novel non-antibody based binders, recombinant therapeutic and non-therapeutic enzymes, virus like particles (VLPs), peptides (hormones and others), monomers of biopolymers, affinity ligands and others. The proteins were mostly monomeric with the size ranging from 2 to 40 kDa. Highest expression titres are obtained in the case of cytoplasmic soluble expression (C2 in Figure 10) with a median titre of 11 g/l culture broth and a range of 3 - 20 g/l. Intentional intracellular expression of recombinant protein in an insoluble state as inclusion bodies (C1 in Figure 10) resulted in a median titre of about 9 g/l, with a range of 3 - 15 g/l dependent on the target protein.

**Extracellular expression, periplasmic and into cell free medium (categories C3 and C4).** Products that were expressed with a signal sequence were fragment antibodies (Fab), Fab fusion proteins, single chain antibodies (scFv), growth factors, enzymes and various formats of amphipathic proteins. The size of the corresponding products varied between 20 and 220
kDa. Among them were both soluble and fairly soluble monomers and multimers, homo- and heteromers. Extracellular product (C3 in Figure 10) reached concentrations in the range of 0.5 to 8.5 g/l in the cell-free medium with a median of 1.5 g/l. Proteins which accumulated in the periplasm (C4 in Figure 10) reached titres of functional product between 0.5 and 10 g/l with a median titre of 2.0 g/l. Dependent on the product-specific aggregation propensity sometimes significant amounts of precipitated recombinant protein were observed in the periplasm. This fraction has been ignored, since it does not contribute to functional product. The extent of product precipitation can be influenced by the choice of the promoter system, the related induction mode and fermentation conditions. Similarly, the distribution of product between the periplasm and the cell-free medium can be partly controlled by changes in physical and chemical environmental conditions. However, ideal conditions need to be identified empirically.

The above mentioned product titres have been typically obtained within 36 to 72 hours of fermentation.

4.5 Posttranslational modification in *Escherichia coli*

Proteins often require posttranslational modification in order to gain full biological activity. Therefore, missing posttranslational protein modification capabilities such as glycosylation, formation of pyroglutamic acid at the N-terminus, N-terminal acylation and C-terminal amidation are frequently cited as a disadvantage of bacterial expression.

However, over the last decade big advances have been made in understanding glycosylation mechanisms and in glycoengineering of microbial organisms. Gerngross and coworkers (Choi et al., 2003) and Contreras and coworkers (Vervecken et al., 2004) were among the first to succeed in glycoengineering yeast more precisely, *Pichia pastoris*, towards the formation of defined glycoforms. The yeast-related work culminated in successful expression of human-like glycosylated antibody in a *Pichia pastoris* host, that enables specific human N-glycosylation with high fidelity (Potgieter et al., 2009).

In parallel it became evident that protein glycosylation is also abundant in prokaryotes. Whereas N-linked protein glycosylation is the most abundant posttranslational modification in eukaryotes, within prokaryotes it seems to be restricted to the domain of the Archea where S-layer proteins show N-linked glycosylation. Already in 2002 Aebi and coworkers (Wacker et al., 2002) demonstrated successful transfer of the *Campylobacter jejuni* protein N-glycosylation machinery into *Escherichia coli*. This opened up an exciting opportunity to produce N-glycoproteins within bacterial expression platforms. Nevertheless, two features were inhibitory to a broad application of the new system. (a) The *Campylobacter jejuni* glycan is immunogenic for humans. (b) The glycan is linked to asparagine through an unusual deoxysugar, bacillosamin. Recently the system has been further developed towards formation of the required N-acetylglucosamin-asparagine linkage that is commonly found in glycoproteins of eukaryotic origin (Schwarz et al., 2010). The same paper proposes a semi-synthetic approach towards human glycosylation based on the new developed technology.

The goal of any microbial glycoengineered system must be to overcome the weaknesses of the existing mammalian platforms that are, (a) mammalian glycosylation is characterized by
naturally occurring heterogeneity in the glycan structure and (b) by limited possibilities to tailor glycosylation towards improved therapeutic performance. Consequently microbial glycoengineered expression platforms should allow for tailored, homogenous and human-like glycosylation. However the challenges on the way to the development of a well performing microbial glycoengineered platform are manifold. The following lists the technical obstacles that need to be addressed:

1. glycoform homogeneity, ideally one glycoform should be formed
2. tailoring, access to a number of specific glycoforms through defined host backgrounds
3. productivity, volumetric productivity should not be below the productivity of existing mammalian systems
4. O-glycosylation, existing yeast O-glycosylation causes immunogenic reactions with humans
5. glycosylation efficiency, the whole of the target protein is expected to be glycosylated
6. secretion efficiency, needs to be high, since glycosylation is connected to secretion
7. expression of complex proteins such as antibodies, capability to produce hetero-multimers (disulphide bridges)
8. plug and play, access to stable glycoengineered hosts, such that only the target gene needs to be inserted
9. proteases, deletion of all undesirable proteolytic activity
10. good growth characteristics, system viability is affected by the amount of genetic changes
11. N-terminal variability, often seen in yeast systems, needs to be under control

As mentioned before, existing mammalian expression technology is not fulfilling all of the desirable requirements and there is an even longer way to go for the existing yeast systems in order to compete with mammalian systems. Not all of the above mentioned technical challenges have been successfully addressed in yeast. Even further away from technical maturity are bacterial glycoengineered systems. Nevertheless technical advances are achieved at high pace. The authors would not be surprised if bacterial expression technology would one day be a viable solution for large scale manufacturing of glycoproteins.

4.6 Cost considerations

From a commercial point of view, bacterial and yeast systems share many advantages over mammalian systems such as high growth rate, the potential to reach high biomass concentration, structural and segregational robustness and a higher product production rate $r_p$, resulting in significantly shorter fermentation times. While mammalian cells such as CHO cells are characterized by a high specific product production rate $q_p$, volumetric productivity $Q_p$ is typically negatively affected by a relatively low growth rate and more importantly by the lower achievable biomass concentration as compared to *Pichia pastoris* (Kunert et al., 2008). The same is true, when comparing CHO cells to *Escherichia coli*. Other aspects such as time required for the development of a stable CHO cell line and media costs should be considered as well. All these aspects add to the attractiveness of microbial and yeast systems when the manufacture of aglycosylated non-antibody type of recombinant proteins is considered. Table 10 shows cost drivers in fermentation of the current key biopharmaceuticals production platforms.
Characteristics driving USP cost | Bacteria | Yeast | Mammalian Cells
--- | --- | --- | ---
Growth rate $\mu [1/h]$ | 0.7 | 0.2 | 0.02
Final dry biomass concentration [g/l] | 60-70 | 80-100 | 3-8
Typical duration of fermentation [days] | 2-3 | 4-5 | 15-20
Specific product production rate $q_P [g/gh]$ | 0.002 | 0.001 | 0.005
Volumetric productivity $Q_P [g/lh]$ | 0.10 | 0.05 | 0.01
Medium cost | low | low | high
Strain development cost and duration | low | medium | high
Equipment standard | steel | steel | steel, disposable

Table 10. Comparison of bacterial, yeast and mammalian system characteristics which drive cost of goods in fermentation; 1) the figures have been modelled based on typical production key figures and assuming an equal product titre of 5 g/l at the end of the fermentation; USP, upstream processing.

Methylotrophic yeast fermentation can be very demanding on equipment performance as a result of the high oxygen demand, high cooling requirements and explosion-proof design because of methanol feeding. Corresponding bioreactor layout requirements are described by Hoeks et al. (2005).

Figure 7 shows that about 9% of all recombinant DNA products are supposedly manufactured with transgenic animals, avian cells, insect cells and viral platforms. On top of these, there are early projects of recombinant expression in plants, filamentous fungi, plants and protozoa. The decision to opt for one of these systems is mostly driven by specific product aspects, cost or IP reasons in order to gain freedom to operate. A cost advantage through higher productivity or lower depreciation compared to more conventional systems is not obvious. Cost allocated to fermentation is typically in the range of 30% to 50% of overall manufacturing costs. Irrespective of the recombinant biosynthesis method used, the DSP costs remain. Therefore the sometimes cited 10X overall cost improvement through the use of one specific expression system and the related USP production platform is difficult to understand if not unrealistic.

The cost of downstream processing (DSP) is more or less independent of the chosen system, if we assume product localization in the cell-free medium. When using Gram-negative expression technology special attention needs to be paid to endotoxin removal. On the other hand a mammalian system makes viral clearance mandatory.

Intracellular production obviously requires cell disruption or product release from the cells followed by a usually more complex biomass removal step. The latter is more or less standardized for conventional expression technologies. Other operations such as inclusion body isolation and purification followed by protein refolding typically drive DSP costs up. Theses higher costs for DSP can only be justified through higher upstream productivity as shown in Figure 10 or a lack of production alternatives. It is also obvious that no significant cost advantage is to be expected on the DSP side, if product needs to be extracted and...
purified out of whole plants. However, in the latter case a significant cost advantage arises if for example, a therapeutic or a vaccine is administered through oral consumption of the whole plant or a non-purified low-cost plant extract.

Please note that other costs for so called secondary manufacturing (e.g. fill and finish, formulation) accrue for the finished product, which we can not discuss here.

5. Conclusions

The industry has become very conservative, risk averse and reluctant to change established and successful manufacturing platforms because of a very strict interpretation of regulatory guidelines. This is also the main reason why the authors think that the main load of biotechnological manufacturing production has remained with the already industrially established microbial (E. coli, yeast) and mammalian production systems and will continue to do so. Nevertheless, regulatory government bodies do welcome novel manufacturing methods for the production of affordable pharmaceuticals because of ever increasing health care costs. Indeed, it cannot be denied that cost pressure and novel applications will help to disturb the established situation. We consider two alternative expression systems to have some potential.

1. Transgenic plants have the possibility to combine therapeutic with nutrition needs. The production of edible vaccines for human or veterinary applications for example appear to be an attractive option especially as the active crop can be phototrophically and cheaply grown locally.

2. Due to their short doubling times and easier cultivation, protozoa offer themselves as a possibility between microbial and mammalian cell culture. Insect cell culture seem to be not as attractive as protozoa as they do not grow as fast and the frequently used BEVS results in more complex isolation and purification procedures.

These two options, however, will again be hampered by another expected or even partly realised breakthrough: the successful targeted humanised glycosylation in yeast and later in bacteria. On top of that, we will sooner or later experience the realisation of extensive pathway engineering and synthetic biology principles, where production organisms will be designed using engineering principles as in the automotive or aerospace industry. It is even harder to imagine how and where alternatives such as plants or protozoa can beat such advanced microbial or mammalian options.

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