

About concrete, stainless steel and microbes

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THE PLANT – THE KEY TO MANUFACTURING

The unmatched capability of the natural biosynthetic machinery for the production and assembly of small and large chiral molecules and therapeutic proteins is increasingly used in the life science industries. In early development stages, Lonza recognised the tremendous potential of biological tools for sustainable manufacturing and has been active since the early days of these developments (Table I).

Our manufacturing tools encompass recombinant microbial fermentation and mammalian cell culture, where we offer our customers services as strain design, process development, process intensification and manufacturing, including regulatory support. Lonza is also the world's largest manufacturer of peptides by solid and liquid phase chemical synthesis. This means that Lonza is able to evaluate and select the optimal route for a given

peptide/protein, and try several options before deciding and developing the optimal solution to establish a commercially viable production process.

Over the last 25 years, Lonza has set up and operates several world-class state of the art production sites in Switzerland, England, Czechia and the US (Figure 1). Moreover, Lonza has established a joint venture with BioOne Capital to design and build Asia's most advanced mammalian cell culture manufacturing facility in Singapore (Figure 2). The facility with a capacity of four times 20,000 l fermentors for mammalian cell fermentation including the necessary DSP suites will accommodate up to 300 employees and be in operation in 2009. All sites (except the site in the Czech republic) specialise in the production of injectable therapeutic proteins and monoclonal antibodies produced either by mammalian or by microbial cell culture, complying with the appropriate FDA requirements for the manufacture of such products.

Table I – This table summarises all technologies available today that could theoretically be used for the production of proteins and/or fine chemicals, and which ones are established and routinely used for commercial production (✓). The table shows also in which categories Lonza is offering its services

	LONZA	Established	Therapeutic Proteins	Fine Chemicals
Prokaryotes	✓	✓	Lonza	Lonza
Yeasts	✓	✓	Lonza	Lonza
Actinomycetes	✓	✓	Lonza	Lonza
Plant cell culture	no	partly	no	no
Trangenic plants	no	partly	no	no
Insect cell culture	no	yes	no	no
Mammalian cell culture	✓	✓	Lonza	no
Transgenic animals	no	partly	No	no

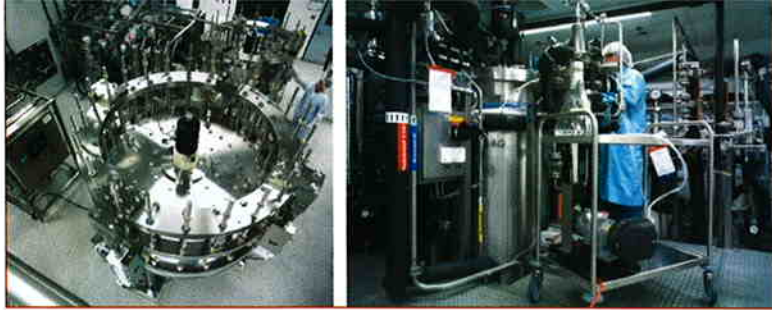


Figure 1 – The photographs show Lonza equipment used in the production of therapeutic proteins. A large chromatography column on the left, and the up-stream area with a 'pilot' scale fermenter illustrate the dimensions



Figure 2 – Lonza mammalian cell culture facility presently being constructed in Singapore, with a fermentation capacity of four times 20,000 litres

THE PRODUCTION STRAIN – THE KEY TO SUCCESS

Money can buy buildings in concrete, shiny and sophisticated plants in stainless steel and top of the line measurement and control systems using the latest computer technology. But one thing remains the key in bioprocessing: the mammalian cell line or microbial strain used in the process, which is the most important ingredient. **The strain is not everything, but the most modern plant is worth nothing without a good production strain.** But these strains have to be designed according to blue prints which follow different rules to civil, mechanical and chemical engineering. In an earlier article entitled "From the test tube to the 75 m³ bioreactor" (1) we gave an overview of what it takes to bring a microbial production process from the stage of designing a strain to production in large fermenters. The article showed how large scale manufacturing with biological systems can reach its limit because of the finely tuned biochemical machinery. But, it was also mentioned that the choice of the production strain is the most critical step since all process inconveniences can be directly or indirectly traced back to the production organism used. For this reason, Lonza has acquired experience in defining and manipulating the characteristics of optimal prokaryotic and eukaryotic production strains, and we are continuously working and testing various microbial platforms for protein production (therapeutic proteins, therapeutic and catalytic enzymes) production (Table II).

THE LONZA EXPRESSION SYSTEM

While our GS expression system for mammalian cells covers the majority of needs for recombinant protein expression in mammalian cell culture, the situation is different in protein expression in microbial cells. There are numerous combinations of hosts and expression systems available,

and the microbial systems that can be used go beyond those listed in Table II. These combinations of hosts and expression systems are also needed, because different applications need different solutions for the production of therapeutic proteins and enzymes. For this reason in addition to the glutamine

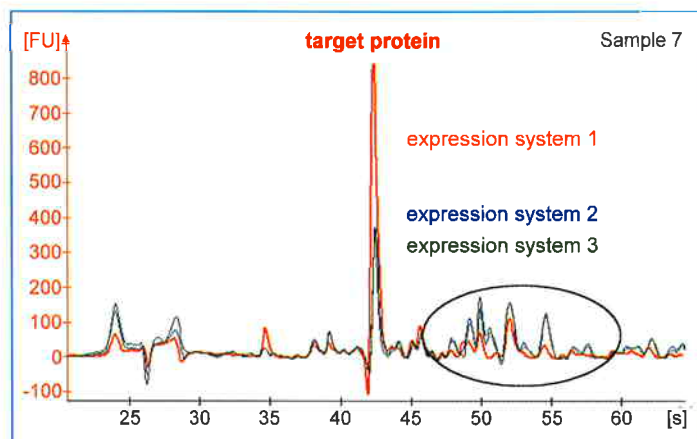


Figure 3 – The protein background (impurities) strongly depends on the choice of the expression system. This Agilent 2100 Bioanalyzer chromatograms show the protein content of different crude extracts of one strain (*Escherichia coli*) producing the same target protein but with the help of different expression systems. This figure illustrates that the quality of the protein extract that is used for purification development is strongly dependent on the expression system and induction conditions

synthetase (GS) expression system for mammalian cell lines, we are working on the development of additional prokaryotic expression systems and combinations. One of our goals is that the optimised expression system has an increased ratio of target protein (product) versus non-product proteins produced. This in combination with the defined medium requirements of the strain, can result in a simple standardised down stream processing procedure. Figure 3 shows the performance of different expression systems in the same host, in this case *Escherichia coli*.

Even expression in the same host but using different promoters for exactly the same target gene results in completely different expression patterns as shown in Figure 4. In the example shown in

Figure 4, the best result was actually achieved with the new Lonza expression system. However, with another protein, the picture may change completely. Predictions are difficult, and an experimental approach by simply comparing different expression systems is quite often the only way to find out the best combination for the production organism.

It is not surprising that our most important workhorses are *E. coli* and *Pichia pastoris*. Yeast cells and particular methylotrophic *Pichia sp.* or *Hansenula sp.* strains are gaining importance for the production of therapeutic proteins and enzymes because of a combination of features such as

- Unicellular GRAS organism;
- Easy cultivation and simple nutritional requirements facilitate high cell density fermentation;
- Secretion of functional proteins into the culture medium;
- Easy purification from the culture medium;

Table II – Overview of microorganisms where Lonza has applied genetic engineering methods for strain development. The table shows also, how important outsourcing is. The list shows that we have concentrated our strain design on unicellular organisms

Organism	Expression system
GRAM-NEGATIVE	
<i>Escherichia coli</i> (<i>E. coli</i>)	in house (IP)
<i>Escherichia coli</i> (Quiescent cells)	evaluation agreement
<i>Pseudomonas putida</i> (and related bacteria)	in house (IP)
<i>Agrobacterium</i> (and related bacteria)	in house
GRAM-POSITIVE	
<i>Brevibacillus choshinensis</i>	evaluation license, license under discussion
<i>Bacillus subtilis</i>	academic cooperation
<i>Lactococcus lactis</i>	evaluation license
METHYLOTROPHIC YEASTS	
<i>Pichia pastoris</i>	license
<i>Hansenula polymorpha</i>	license under discussion

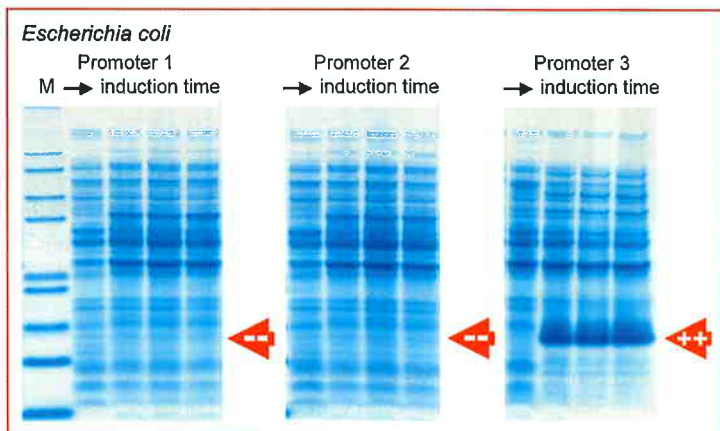


Figure 4 – Promoter specificity of the heterologous expression of a target gene. The target gene was integrated into different expression plasmids for *Escherichia coli* which contain different inducible promoters. After shake flask induction experiments, protein extracts were analyzed by SDS-PAGE. Only promoter 3 expresses the target gene.

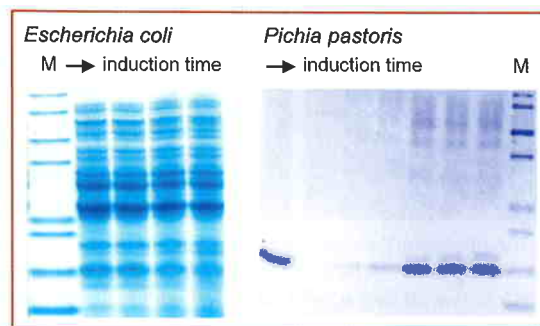


Figure 5 – Host specificity of the heterologous production of a target protein. The amino acid sequence of a target protein was used to derive codon-optimized genes for *Escherichia coli* and *Pichia pastoris*. The genes were integrated into different expression plasmids and used to transform both strains. After shake flask induction experiments, protein extracts were analyzed by SDS-PAGE.

- Post-translational modifications possible if necessary.

At Lonza several different therapeutic and other peptides were produced in *Pichia pastoris*. In these cases, the choice of the host for the heterologous expression of a target protein had to be experimentally determined. Expression in different hosts can be quite different. However comparing the expression of the same protein in *E. coli* and yeast, one can notice considerable differences (Figure 5). While *Pichia pastoris* is producing the target protein as shown on the right gel with a strong protein band, *E. coli* is producing a variety of different proteins but not the target protein (see the numerous protein bands on the left gel).

(Figure 6) or other aspects increasing manufacturing costs either in fermentation and in down stream processing. For example: when producing a recombinant protein with *Pichia pastoris*, product variants can be frequently observed as a result of incomplete N-terminal processing and C-terminal truncation.

Thus, after all these comments about the strain we have to come back to the concrete and the stainless steel. Because of such high biomass concentrations, high oxygen uptake rates (5-10 mmol/g/h), high nutrient demand and high heat evolution – the design of the equipment has to be accordingly. For this purpose, one of the networks LONZA has established is a cooperation programme between the Technical University in Prague (Czechia,

Prof. K. Melzoch), the University of Applied Sciences in Wädenswil (Switzerland, Prof. K. Kovar) the Lonza manufacturing site in Kourim (Czechia) and the microbial R&D department in Visp (Switzerland) to address the process engineering questions for highly productive microbial processes. After all, it is not the concrete, not the steel not even the highly praised strain which is ultimately making the difference. It is the human brain elaborating innovative approaches to protein production problems.

REFERENCE

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PROCESS INTENSIFICATION – THE KEY TO COST CONTROL

Once a strain construct, the tool for value creation is completed, the most important step has been realised. What remains to be done was also described in our previous article (1), is not an easy task either. It can be described as process intensification, with the goal to maximise productivity during fermentation, reduce the isolation and purification unit operations (e.g. by integrating process steps) and to reduce losses in the different steps. Nowadays, microbial fermentations are carried out in high cell density mode, which means that very high biomass concentrations (several hundred grams of dry weight per litre fermentation broth) are achieved.

Like any other horse, our microbial 'workhorses' *Escherichia coli* and *Pichia sp.* have the tendency to 'bolt' under these high cell density culture conditions, which are accompanied by unfavourable conditions if not properly controlled. Oxygen limitations, heat transfer limitations and nutrient limitations may occur under these circumstances, resulting in reduced product biosynthesis, by-product formation

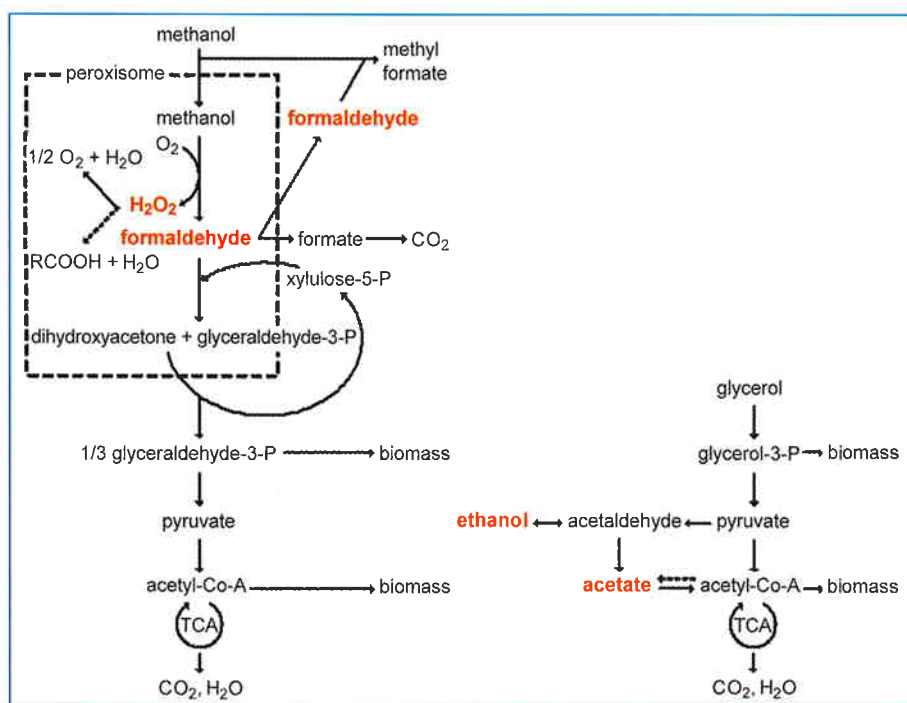


Figure 6 – This scheme shows in red the toxic or at least inhibiting substances that can accumulate when growing, for example, a methylotrophic yeast *Pichia pastoris* on methanol as substrate (left) or glycerol as substrate (right). Needless to say that *Escherichia coli* accumulates similar inhibiting by-products (ethanol, acetate, succinate) when grown at high cell density on, for example, glucose as substrate