



Meeting report

Recombinant Protein Production 6 (Vienna, February 2011)

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The sixth edition of the Conference on Recombinant Protein Production saw a return of physiology-based cell and process engineering. While the application of omics technologies to cell engineering has been constantly on the rise during the past decade, the concept of systems biotechnology is now also applied on bioprocesses bringing new insights into process design and production strategies. The conference brought an extensive comparative view on host cell physiology, covering all areas of bacterial, yeast, fungal, insect, plant and mammalian protein production hosts. Global (genome scale) cellular analysis led to local cell engineering strategies covering also interspecies host optimization strategies, and bringing energy requirements during recombinant protein production back into focus. Additionally, the development of novel secretion systems was presented, giving one example of how to combine industry's needs with highly ambitious fundamental research.

Since 2000, this meeting series organized by the Microbial Physiology Section of the European Federation of Biotechnology developed to a very popular event, attracting over 250 participants every second year. The latest edition was held from 16th to 19th February 2011, in Vienna, Austria, and organized by an international team led by David Archer (Univ. Nottingham, UK) and Diethard Mattanovich (BOKU Univ. Natural Resources and Life Sciences Vienna) with great support of numerous colleagues of BOKU and the Austrian Centre of Industrial Biotechnology

(ACIB). FEMS provided grants for speakers and young scientists to participate. Novozymes donated three generous cash prizes for the best posters by young scientists. Sponsorship by Sandoz, Boehringer Ingelheim, VTU Technology, DASGIP, Cellon, Sanofi-Aventis and AstraZeneca is also gratefully acknowledged.

Keynote lectures highlight stress responses

The meeting was divided into six sessions spanning from quality control over production of

multimeric proteins to strains and processes. Metabolism and regulation of recombinant protein production were touched and finally the topic of protein secretion was addressed. Two keynote lectures highlighted the trend to high-level cell physiology research in the field of protein production. Anne Willis (Univ. Leicester, UK) opened the conference with an introduction into stress-related post-transcriptional control of gene expression. Upon stress ribosomes are specifically recruited to mRNAs needed for stress response, thus shutting down most protein synthesis. Randal Kaufman (Univ. Michigan, USA) highlighted the link of unfolded protein response with oxidative stress in mammalian cells, a topic with wide outreach to other eukaryotic, maybe even prokaryotic host systems.

Biological quality control

The first session was opened by Diethard Mattanovich (BOKU Vienna, AT) who underlined how stressful recombinant protein production is for *Pichia pastoris*: environmental influences, which are process-related conditions, have a strong impact on protein production. However, protein overproduction itself challenges the cells internally, because protein synthesis and folding demand energy. Contrary to what has been believed for a long time this energy demand is not negligible compared to the overall energy demand of the growing cell. Consequently, the TCA cycle has a major role for heterologous protein production – an observation, which has been repeatedly highlighted throughout the meeting in context of different host organisms.

Another yeast expression system, for which major steps forward have been presented, is *Yarrowia lipolytica*. Wouter Vervecken (Oxyrane, Gent, BE) gave an overview of the production of recombinant proteins for lysosomal storage

disease (LSD) enzyme replacement therapies. Such biologics require a mannose 6-phosphate carbohydrate for effective cellular targeting and subcellular localization. However, this is a rare and rather unstable protein modification leading to low productivities in mammalian cell lines. Oxyrane's presented answer to this problem is the successful expression of these proteins in a glyco-engineered *Y. lipolytica* strain. While the product titer is comparable to the titer reached with a mammalian cell system, up to 80% of the produced therapeutics are correctly modified, which is a major step forward for the treatment of these diseases. It proves furthermore that glycoengineering of yeast strains is a promising endeavor and that the crossing of species boundaries can be highly advantageous. Another approach of host optimization crossing species boundaries was presented by Neus Ferrer-Miralles from the Autonomous Univ. Barcelona in Spain. She showed that the prokaryotic DnaK/DnaJ chaperone pair can be successfully rehosted in eukaryotic expression systems as folding modulators. Strikingly, the presence of the bacterial chaperones in fact increases protein solubility, stability and conformational quality of recombinant proteins produced in insect cell- or larvae-baculovirus expression systems.

Difficult to produce proteins were the targets presented by Jan Maarten van Dijk from the University of Groningen (NL) and Harald Pichler (ACIB and Graz Univ. of Technology, AT). Jan Maarten van Dijk showed that a disruption of the secretion sensing system CsrRS allows the overproduction of membrane proteins which could not be produced in *Bacillus subtilis* before. Harald Pichler set out to produce alternative pig liver esterase as valuable biocatalyst for industrial processes. Also in this case a bacterial system (*Escherichia coli*) was the overall winner in a race for production hosts. However, the final kick for significant overproduction was a modification of the enzyme itself. Abolishing the multimeric nature of the enzyme in favor to a monomeric state improved the expression rate remarkably.

Multimeric proteins

Multimeric nature was the topic of the following session that was initiated by Antonio Villaverde from the Autonomous University of Barcelona (ES). Sticking to bacteria as production hosts, he showed the fascinating selfassembly of virus-like nanoparticles which could be useful as DNA vectors for gene therapy. Wolfgang Buchinger from Boehringer Ingelheim in Austria presented an enlightening comparison of industry's

experience with three expression hosts: *Pseudomonas fluorescens*, *E. coli* and *P. pastoris*. The conclusion was, that we are still far from a general preference for one expression system for the production of Fab antibody fragments. We are still in a period where it appears worthwhile to evaluate several microbial systems for production of a certain protein in parallel, and where experimental work has to be performed to analyze which host system is the best suited cell factory for each single protein, even if just one class like antibody fragments is in focus. By contrast, Anne Tolstrup from Symphogen A/S (DK) presented a clear favorite for production of antibody mixtures for therapeutic uses. It has been possible to establish cell lines with such a remarkable clonal stability, that the cell lines can be mixed in a desired ratio to obtain a desired mixture of antibodies after a single fermentation step. FDA approval of a production line employing a master cell bank comprising such a mixture of clones secreting different antibodies marks a milestone for the development of biotechnology.

Production strains and processes

Michael Rasmussen (Novozymes, DK) summarized more than 50 years history of the protein production system *B. subtilis*, including the developed toolboxes (screening, mutagenesis of enzymes and activity optimization of proteins), which are the reason that *Bacillus* is one of the most used hosts from university to industrial applications. The high potential of *Bacillus* sp. for recombinant protein expression is also reflected by the development of the molecular tools for the species *Bacillus amyloliquefaciens*, including plasmids with two strong promoters (one inducible) and antibiotic free selection, as demonstrated by Stefan Schöner (c-LEcta, DE).

Protein features like solubility, aggregation or membrane targeting interact with the production host and the final use. The yeast, *Saccharomyces cerevisiae*, has been chosen by Stephanie Cartwright for the production of membrane proteins. Several knockout strains showed a different polysome and ribosome stoichiometry, causing an increase of membrane protein yield. A systematic evaluation of a fusion system for protein production in *E. coli* demonstrated that the fusion tags, Fh8 and H, increased the yield and the specific immunogenicity of five pharmaceutically interesting immunogenic proteins compared to the native proteins (Sofia Costa, Univ. Minho, PT). Jesús Zurdo (Lonza, UK) presented an elegant example of a bioinformatics approach for

process improvement by a predictive algorithm for protein aggregation and stability during initial screening for therapeutic candidates. A significant limitation of higher eukaryotic expression systems is development time, addressed by two presentations. Jens Wölfel (CEVEC Pharmaceuticals, DE) explained how one can arrive from transfection to production within 2 weeks with mammalian cells by using CAP-T-cells and transient DNA transfer. Emilio Casanova (LBI Cancer Research, AT) presented the use of Bacterial Artificial Chromosomes for stable, high-level expression of recombinant proteins in mammalian cells, because of the capacity of the integration of host mammalian loci and high copy numbers.

As an example of physiology-based process control Moira Schuler (Dublin City Univ., IE) suggested the multivariate analysis of cultivation variables, such as off gas composition and spectroscopic measurements (FTIR), for a feed back control of fed batch cultivation of *E. coli*, *S. cerevisiae* and *P. pastoris*, thus improving the process stability.

Genome scale regulation: transcription, translation and cell metabolism

Metabolite profiling and gene regulation patterns were used as read-outs of the quality of industrial production processes, and for early prediction of positive cellular traits for rational cell and process engineering in sessions 4 and 5, respectively. Metabolomics (session 4) reflect the combination of gene expression and cellular environment, whereas transcriptomics and proteomics (session 5) are a steady readout of cell status.

Alan Dickson (Univ. Manchester, UK) characterized critical metabolites in IgG4 producing GS-CHO and their responsiveness to media feed. Thereby it was observed that many commercially available CHO media are too rich, and lead to a waste of glucose. Rational feed design based on these observations resulted in a media consisting of just four components, which improved growth, viability and consequently product titer. Niall Barron (Dublin City Univ., IE) introduced micro-RNA (post-transcriptional gene regulation mechanism) as targets for CHO cell line development. Identification and engineering of miR-7 expression, which is a micro-RNA positively involved in proliferation control, resulted in a high level of viable cells after seven days, and increased productivity up to tenfold. Another crucial issue in CHO cell culture is the requirement of glutamine, as the release of ammonium has an impact on glycosylation and protein quality. A combined

approach of directed evolution and cell sorting was applied by Nicole Borth (BOKU Vienna, Austria) to obtain a glutamine-independent recombinant CHO cell line. By analyzing mRNAs and microRNAs, a cross-correlation of miRNA expression to mRNA expression was found, and the differential regulation of central metabolic pathways in glutamine-independent cells was revealed. The final aim was to identify markers that reflect the state of the cells in a bioreactor.

Energy and core metabolism were newly identified as limiting factors for recombinant protein production in yeasts, as studied by ^{13}C -based metabolic flux analysis. High-level RPP was correlated to an increased flux through the TCA cycle in *P. pastoris*, thus indicating increased energy demand and metabolic limitation, both during production of intracellular as well as secreted recombinant proteins. While Lars Blank (Dortmund Univ., DE) proposed to overcome this limitation by adding amino acids, Pau Ferrer (Autonomous Univ. Barcelona, ES) suggests the engineering of strains with reduced energy requirements. In this context, the EnBase Technology (BioSilta, FI) may offer a high throughput screening technique for various cultivation parameters or media optimization. A comparative transcriptome and proteome analysis of the effect of oxygenation on recombinant *S. cerevisiae* and *P. pastoris* was presented by Laura Dato (Univ. Milano-Bicocca, IT). Hypoxic conditions favor secretion of Fab fragments in *P. pastoris*, but not in *S. cerevisiae*, and are accompanied by very distinct remodeling of gene expression in the two hosts. Major differences in regulation of central carbon metabolism, cell stress and unfolded protein response give hints for strain improvement and may assist in the choice of the appropriate host.

Central carbon metabolism is also of major interest during RPP in bacterial cells. Ursula Rinas (Helmholtz Center for Infection Research, Braunschweig, DE) studied the impact of catabolic response on RPP in *E. coli* upon IPTG induction, and identified precursors for protein synthesis to be strongly downregulated at high growth rates. Overloading of *E. coli* metabolism by RPP was also reported by Florian Strobl (ACIB and BOKU Vienna, AT), who applied transcription rate control based on feeding limiting amounts

of IPTG in a constant ratio to biomass or plasmid-free genome integrated expression systems to improve both cell growth and soluble protein yield. In agreement with these results, Jeff Cole (Univ. Birmingham, UK) found mutations down-regulating expression of T7 polymerase in all BL21 strains, which were isolated for having improved RPP based on cell sorting of the recombinant protein fused to GFP. On the other hand, improvements of RPP were achieved by increasing biomass yield of recombinant *E. coli*. The knockout of two regulators of acetate formation (*iclR* and *arcA*) by Henrick Waegeman (Gent Univ., BE) increased TCA cycle flux and cytosolic protein production and lowered acetate formation in K-strains, but had no effect on BL21, which already possesses higher basal TCA activity and an active glyoxylate shunt. This difference was also observed by Joseph Shiloach (NIDDK/NIH Bethesda, USA) who reached lower acetate excretion by limiting glucose uptake rates through the overexpression of the small regulatory RNA SgrS.

Novel industrial approaches to overcome bottlenecks for RPP in *Aspergillus* were covered by Herman J. Pel (DSM Biotechnology Center, NL), and included codon pair bias optimization and a classifier approach correlating protein structural features to expression levels. The sessions were closed by Bernhard Seiboth (Vienna Univ. Technology, AT), who introduced epigenetic regulation to the field of RPP using the control of cellulase gene expression in *Trichoderma reesei* as an example.

Protein secretion

Secretion plays a major role in protein production as it enables high purity and quality of the product. However, protein secretion is often limited because of degradation and slow folding and transport kinetics. Arthur Ram (Leiden Univ., NL) demonstrated the importance of ER associated degradation (ERAD) in *Aspergillus niger* and Nicolas Mermoud (Univ. Lausanne, CH) introduced novel concepts for homologous recombination and folding pathway engineering in mammalian cells. The quantitative importance of degradation and secretion kinetics was highlighted by Martin Pfeffer (BOKU Vienna, AT) who showed that 60%

of a recombinant antibody fragment was degraded in *P. pastoris* before secretion. Novel secretion strategies were introduced by Jussi Joensuu (VTT, FI) using hydrophobin fusions to trap proteins in ER-like vesicles in filamentous fungi and plants. The meeting was closed by Ian Henderson (Univ. Birmingham, UK) who demonstrated an elegant application of a bacterial autotransporter system.

Perspectives

Industrial production of recombinant proteins faces important challenges today: many new biopharmaceuticals like monoclonal antibodies are administered at high doses, requiring large amounts of these drugs at reasonably low price. White biotechnology, on the other side, will require huge amounts of cheap enzymes for modern biorefinery concepts. Although often claimed to be more art than science, recombinant protein production still needs high-level fundamental research to enable industry to reach these current goals both in biopharmaceutical and white biotechnology: high product titers in short time and high uniform product quality, achieved in simple integrated processes. Physiology of the producing host is a key to achieve these goals. We are about to understand how to implement genome scale datasets derived by omics techniques into cell biology research, leading us to targets for cell engineering. Exciting times lie ahead of us as we approach a 'whole cell' description of protein production, but we need to be aware that the expectations and demands of industry are high.

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