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Meeting Report

Recombinant protein production: a comparative view on host physiology

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The fifth meeting organised by the EFB on the influence of host physiology on recombinant protein production was held in Alghero, Sardinia in September 2008. Conclusions from the meeting included that a very wide range of hosts are still needed to produce recombinant proteins of quality adequate for use in human healthcare. CHO cells can produce excellent titres, but it remains impossible to predict how a transfection line will perform, especially at high cell density when productivity declines: *Pichia pastoris* might replace mammalian cell cultures for many applications. A series of transcriptomic and proteomic studies have generated large datasets that provide a valuable resource for understanding how to improve recombinant protein production, but this remains a promise rather than a *fait accompli*. *E. coli* remains the workhorse for over half of the recombinant proteins produced by the biopharmaceutical industries. Until recently, its Achilles heel was its inability to glycosylate eukaryotic proteins, a problem for which the development of bacterial N-linked protein glycosylation systems offers an imminent solution.

The fifth meeting in this highly successful series organised by the Microbial Physiology Section of the European Federation of Biotechnology was held from 24th–28th September 2008, in Alghero, Sardinia, and expertly organised by Enrico Berardi (University of Palermo, Italy), Gloria Muzzi-Erichsen (Novozymes, California) and their colleagues. FEMS provided grants for speakers and young scientists to participate; Novozymes donated three generous cash prizes for the best posters by young scientists and with Ursula Rinas organised the selection of the best posters; The ESF sponsored one of the six sessions on production of membrane proteins; and sponsorship by CMC Biologics, Lonza, Novonordisk and Sanofi-Aventis is also gratefully acknowledged.

The meeting was opened by the Section Chairman, Jeff Cole, who explained the main elements that have made these meetings so heavily oversubscribed. They were the unique combination of participation from industry (80 people at the current meeting), research institutes and academia; inclusion of all types of expression host from bacteria and viruses to plant, animal, human and insect cells; prominent involvement of the bioprocessing industry in coordination with the scientific programme; and the combination of low registration fees and ambiance of the meeting locations. The Section Secretary, Diethard Mattanovich (who was also the Chair of the Scientific Programme Committee), mapped the developments in the field since the start of the series. In 2000, the emphasis in Semmering was on the stress responses due to the metabolic burden of recombinant protein production (RPP) on the host, and how to optimise the link between growth and product accumulation. Energetic requirements of the host dominated the 2002 meeting in Como; glycoengineering in eukaryotes and protein folding were key foci at Tavira in 2004, while exploitation of 'omics' technologies were prominent in the Barcelona 2006 programme. Systems biology was predicted to be a major theme of the current meeting—though all of the previously identified problems were revisited during the intensive four-day meeting.

Coordination of molecular chaperones in the yeast *Saccharomyces cerevisiae*

The meeting opened with a plenary lecture by Colin Stirling (Manchester, UK), who presented a tour-de-force review of developments in the field of protein folding in the yeast,

Saccharomyces cerevisiae. Co-ordinated chaperone activity folds recombinant proteins in the endoplasmic reticulum (ER) from where they are exported to the Golgi apparatus for secretion. Any excess protein that remains unfolded owing to a post-translational bottleneck induces the unfolded protein response (UPR), the outcome of which is endoplasmic reticulum-associated degradation (ERAD). Understanding the mechanism of these alternative fates of proteins is crucial for human health, because some genetic diseases originate from defective protein folding in the ER. Two proteins of the Hsp70 family play crucial roles in these processes: Kar2p is required for protein uptake into the ER, for folding, UPR and ERAD; Lhs1P is required for import and folding. Two co-chaperones are also required: J protein delivers the unfolded substrate to the C-terminal domain of the Hsp70 protein and stimulates the ATPase activity of the N-terminal domain; nucleotide exchange factor promotes release of ADP and release of the substrate from the J protein. Because no ER exchange factor had been identified, until recently a fascinating problem concerned the role of Kar2p in binding unfolded proteins at the protein import site, a key question being how a tightly associated substrate is released from Kar2p once it is in the ER. An early clue to the explanation came from the observation that the UPR is induced in strains defective in a further protein, Lhs1, owing to the accumulation of unfolded proteins, and double mutants defective in both Lhs1 and ire1 are not viable. However, Sil1 suppresses death of the double mutant, implicating it as a second exchange factor that can replace Lhs1. Elegant inducible suppression experiments were described that revealed that a double mutant defective in both Sil1 and Lhs1 is non-viable because of its inability to import proteins into the ER. No structure is currently available for Kar2p, but both Sil1p and Lhs1 bind to Kar2p, and an E348R substitution in Kar2p discriminates between the bindings of the exchange factors. A summary of the recycling pathway for proteins that fail to be folded in the ER was then presented, together with explanation of the role of the nucleotide exchange factor, Lhs1, in the prevention of heat-induced aggregation. This elegantly linked normal protein folding to the stress response.

Transcription/translation control and engineering

The subsequent three full days of the meeting were divided into six sessions. Each session began with an invited lecture, mostly followed by two short presentations selected from the offered papers. After a caffeine break, this pattern was repeated, resulting in an acceptable format that sustained audience attention. Leif Isaksson (Stockholm University) opened the show by reminding us of the importance of codon optimisation when expressing heterologous proteins in *Escherichia coli*, which can generate up to a 10-fold improvement in yield. The range of codons at which translation can start was also reviewed: NUG and AUG are effective; ANG is unacceptable (N is any base). Also emphasised was the codon context not only at the start but also at the termination of translation where translation efficiency depends greatly on context. For example, all of the first six codons affect translation efficiency, with NGG codons being especially detrimental, but optimisation of the second codon has the greatest beneficial effect. Rahmi Lale from the Norwegian University of Science and Technology then reviewed the influence of the 5′ untranslated region of the mRNA on translation efficiency: mutation of hot spots led to a 20-fold increase in β-lactamase synthesis, but also could increase transcription up to 8-fold. These effects are independent of promoter strength or promoter regulation. Later in the session, Noël van Peij (DSM Food Specialities, NL) returned to the theme of codon usage, showing that certain dinucleotide pairs had detrimental effects on translation in the fungal host, *Aspergillus niger*.

Post-transcriptional events also dramatically affect the productivity of CHO cells, a point emphasised by Laura Bailey (Manchester University), who reminded us that the annual market for antibodies was currently running at about $20 billion. When cultured with glutamate to select for glutamine synthesis, yields of up to 5 g l^{-1} can be achieved, especially with fed-batch cultivation, but the productivity of even the best cell lines gradually declines owing not to loss of transcription, but to poor protein folding and secretion. Nic Mermod from the University of Lausanne reviewed how the matrix attachment region elements, MARs, can be exploited to alleviate epigenetic gene silencing and maintain expression in mammalian cell cultures. MARs apparently stabilise a transcription initiation complex, resulting in decreased variability and a shift to higher expression levels. A second round of transfection generates first cointegrated concatamers, and then integration into the CHO cell genome, again resulting in higher expression levels. Productivity can vary in the range 20–120 pg of product/cell/day, and a record yield of 41 g of IgG per litre of culture over a 12-day period was reported.

GFP fusions constructed rapidly by crossover PCR are being exploited by Maria Freigassner (Graz University of Technology) for high-throughput screening of membrane proteins using *Pichia pastoris* as the host. This was the first of many presentations that heralded the maturation of the *P. pastoris* system to challenge less consistent CHO cells or other mammalian cells as the preferred host for mammalian proteins, facilitated by the availability of strong, tightly regulated promoters and ability to generate dense cell cultures. Analysis of the transcriptomic response to high-level RPP revealed the now-familiar gross changes in levels of transcripts for chaperones, folding proteins and proteases. The response varied with the target protein being expressed.

Energy metabolism of heterologous protein production

The second session began with two *E. coli* presentations by Mark Eiteman (Georgia, USA) and Karoline Marisch (Vienna), which were nicely complemented by a presentation by Paula Alves (ITQB, Portugal) on the cell density effect in the baculovirus insect cell system. Key points were a reminder that acetate accumulates prolifically in a nitrogen-limited culture. This can be correlated with an increase in the NADH to NAD ratio above a crucial threshold of 0.06. The glucose effect is abrogated by metabolic engineering to limit NAD reduction, a response that can be correlated with the control of metabolic fluxes mediated by the ArcB-ArcA two components regulatory system that detects and responds to the redox state of the ubiquinol pool. A genomic comparison of two workhorse strains of *E. coli*, K-12 and BL21, revealed that they share 3978 genes in common. Combined transcriptomic and proteomic studies revealed that expression of 228 genes responded to the stress imposed by recombinant protein production—a theme that was to recur in many subsequent presentations.

The baculovirus insect cell system can generate excellent yields of RP, but is limited by the loss of both specific and volumetric productivities at high cell density (another theme that will recur in the context of CHO cells!). On infection, baculovirus metabolism is downregulated—but strangely this is followed by upregulation of the energy status of the cells. Pyruvate, but not glutamine or 2-oxoglutarate, was shown to be beneficial in restoring productivity.

The three last talks in this session explored the link between cultivation conditions and

heterologous protein production in filamentous fungi and yeast. These three presentations yielded contrasting conclusions, raising the question whether yet more environmental factors that affect recombinant protein yields remain to be studied.

Merja Penttila (VTT Technical Research Centre of Finland) demonstrated that heterologous protein production in *Trichoderma reesei* causes increased protein degradation as well as downregulation of transcriptional, translational and secretion functions, the stress response that can be artificially induced by adding DTT to the culture. Highest yields of extracellular protein in chemostat cultures were achieved at lower growth rates (0.03 h^{-1}) and reduced biomass accumulation. Gene expression patterns at different culture growth rates and specific protein production rates revealed a positive correlation with extracellular protein production and a negative correlation with energy metabolism and secretion pathways. Kristin Baumann (Autonomous University of Barcelona, Spain) has shown that, compared with oxygen sufficient growth, a 2.5-fold increase in production of antibody Fab fragment was observed when *Pichia pastoris* was grown at a low oxygen concentration in the inlet air (8.39%; hypoxia). At the low oxygenation rate, optimised fed-batch cultures switched from respiratory to low level fermentative metabolism that is accompanied by the formation of ethanol and C5 sugar alcohols and a decrease in biomass yield. Interestingly, both producing and non-producing cultures showed very similar oxygen-dependent gene expression patterns: upregulation of fungal stress and glycolysis genes; and downregulation of TCA cycle and glycerolipid metabolism genes.

Totally contrasting results with the filamentous fungus *Aspergillus niger*, in which oxygen availability is often a limiting factor in enzyme production, was reported by Karsten Hellmuth (Chr. Hansen Nienburg GmbH, Nienburg, Germany). When the dissolved oxygen concentration was increased either by increasing the stirrer speed or by adding hydrogen peroxide to increase oxygen availability, both the oxygen uptake rate and specific productivity increased, albeit slightly in the latter case. Microarray studies showed that increased stirrer speed induces expression of genes required for cell wall biosynthesis, which indicates shear stress, as well as TCA cycle genes, reflecting increased oxygen uptake and higher metabolic activity. Generally, feeding peroxide had an impact on fatty acid metabolism and gluconeogenesis, but did not induce oxidative stress-related genes, apart from induction of peroxisomes.

Stress response, cellular quality control

Increased recombinant protein expression in *E. coli* is known to induce the general stress response. Expression of membrane proteins is often an additional challenge due to their toxicity to the cell. Jan-Wilem de Gier (Centre for Biomembrane Research, Sweden) reported the development of a BL21(DE3) host suitable for membrane protein expression that emerged from understanding of how membrane protein toxicity is overcome in Walker strains (C41 and C43). These strains show decreased accumulation of mRNA of a recombinant gene as well as negligible accumulation of T7 RNA polymerase. Identification of down-mutations in −10 promoter element in P_{lac} governing the expression of T7 Pol confirmed that a slow rate of product formation is the key to improved membrane protein production in Walker strains. To mimic the effect of these mutations in the engineered BL21(DE3), pLemo plasmid was introduced in which lysozyme, a natural inhibitor of T7 Pol, was provided under the control of a tightly regulated rhamnose promoter. The strain showed robust expression of several membrane proteins that could be manipulated by changing inducer concentration.

The following presentation by Jeff Cole (University of Birmingham, UK) has strengthened the idea even more that slow rates of recombinant protein accumulation combined with lower growth rate result in much better yields of soluble and total protein as well as cell biomass in *E. coli* BL21* (DE3) batch cultivations. The use of very low inducer concentrations (micromolar range) and constant low cultivation temperature helps to minimise or avoid the general stress response, resulting in dramatic improvement of productivity (up to 3-fold) and protein quality (>80% soluble protein). While high inducer concentrations and temperature shift cause severe growth arrest in the typical protein production scheme, in the improved protocol more than 90% of bacteria retain expression plasmid and continue to grow and accumulate correctly folded protein. The process was shown to have a great potential for further intensification (a further 5-fold increase in productivity in fed-batch mode) and application for expression of difficult proteins that require post-translational modifications.

Elena Garcia-Fruitos (Autonomous University of Barcelona, Spain) explored the connection between protein solubility, conformational quality and their genetic control. The fluorescence of GFP has widely been assumed to be a sound criterion for correct protein folding: conversely, inclusion bodies were assumed to accumulate only unfolded, insoluble proteins. The observation that some protein inclusion bodies contain correctly folded protein, as shown by fluorescence of their GFP fusion tag, suggested that protein molecules with different conformational states and hence different biological activities form inclusion bodies. Correctly folded, mis-folded and partially folded protein species are distributed between dynamic soluble and insoluble fractions. The protein molecules are continually removed from the latter for degradation or re-folding by action of chaperones and proteases, such as DnaK, ClpP, ClpX and GroEL/ES factors. Mutation of genes encoding some chaperones and proteases (especially DnaK) coincided with increased fluorescence of the recombinant GFP fusion proteins but also higher protein aggregation rates.

David Archer (Nottingham, UK) described opportunities for comparative analysis between *Aspergillus niger* and *Saccharomyces cerevisiae* of secretion-related genes, using different approaches such as transcriptomics, proteomics and polysomics to provide insights and a better scope for manipulating or engineering host variants that are better secretors. Transcriptome analysis revealed that the protein secretion bottleneck in *A. niger* initiates the UPR and subsequently ERAD responses—both being initiated by the Hac1P bZIP transcription factor. It was emphasised that the mere upregulation of individual chaperones or foldases may not provide improved secretion. First, the level of over-expressed chaperone or foldase may be crucial for some target proteins and second, the coordinated action of several chaperones and co-chaperones may be required. However, a further understanding of the genetic basis of hosts during protein overproduction could provide a longer lasting solution to the problems currently being faced in this field.

Martin Dragosits (Vienna) then provided insights, using consistent findings from transciptome and proteome analysis, of how *Pichia pastoris* responds during antibody overproduction at different temperatures in chemostat cultivation settings. Analysis of the proteome at lower temperatures of 20 °C rather than 30 °C led to the identification of 49 differentially regulated proteins. There was a marked decrease in proteins involved in the oxidative stress response and the specific productivity was significantly higher, which was reasoned to be due to the relief of protein

folding stress—beautifully complementing findings with similar studies with *E. coli* presented earlier in the programme. Such proteome and transcriptome analytical approaches have enabled a detailed assessment of the global cellular regulation responses in both fungi and bacteria, and could outline universal and generic strategies exploiting the reduction of stress to improve protein productivity. Bjorn Hock (Merck, Germany) added another dimension to improving RPP research by outlining an innovative approach of rapidly screening for optimum RPP levels of different proteins at different growth conditions, and using different bacterial hosts. This new technology, based on Luminex xMAP™ beads, uses GFP as a reporter. The accuracy and the increased speed of such a screening method could render it an excellent tool for defining optimum conditions that would increase production of proteins of different properties, and before large proteomic and transciptomic studies could be completed.

Folding, assembly and secretion of complex protein entities

This fourth session was sponsored by the European Science Foundation, represented by Paul Beckers from ESF in Strasbourg. Paul described the unique features of the EUROCORES programme, which is one of the few 'bottom up' opportunities for collaborative research funding across European States. The scientific session opened with a review by Kenneth Lundstrom (PanTherapeutics, Switzerland) of problems associated with the expression of recombinant membrane proteins, which often is a prerequisite for structure-based drug design. Approximately 70% of current drug targets are membrane proteins, so every possible host has been assessed for their ability to express them. Problems include low yields of <1 mg l^{-1} of culture, and their accumulation in inclusion bodies, requiring extensive refolding. Furthermore, expression in membranes require solubilisation applying detergents, which also has a negative effect on yields. Additionally, crystallisation of membrane proteins is more difficult than for soluble proteins. A feasible approach has been to initiate large structural genomics initiatives where a large number of membrane proteins have been expressed in parallel in different expression systems. The expression of G protein-coupled receptors has been complicated although applying Semliki Forest virus vectors for infection of mammalian cells has resulted in large quantities (up to 10 mg l^{-1}). Recently, success has been achieved using the baculovirus system, the most striking examples being the determination of the structures of the human β2 and the turkey β1 adrenergic receptors. These structures will provide new possibilities for structure-based drug design.

Markku Saloheimo (VTT, Espoo) presented the ESF project GENOPHYS, a comparative transcriptomic and proteomic analysis of the production of the Fab fragment, 3H6, in chemostat cultures of five bacterial and fungal systems. While productivities differ widely between these host systems, interesting similarities in physiological reactions to environmental conditions were observed. These presentations were nicely complemented by the final talk in the session by Yoav Peleg (Weizmann Inst., Israel), who reviewed considerable progress in the expression and structure determination, albeit to only 3.5 Å, of a Drosophila adhesion protein, amalgam. This protein interacts with the membrane protein neurotactin. It is a secreted protein with three Ig domains and three predicted N-linked glycosylation sites. It cannot be expressed in a soluble, folded form in *E. coli*, but success was achieved using the *P. pastoris* system—a recurring theme of the entire meeting.

A promising approach to solving some of these expression problems was described by Arnold Driessen (Groningen), who summarised the role of YidC in assisting the SecYEG general secretion translocon in membrane insertion and folding of membrane proteins. Successful exploitation of the homologue, SpoIIIJ, might enable the high secretion capacity of *Bacillus subtilis* to be exploited more extensively, which is a goal of the ESF BACELL Euroscope project. Mark van Raaij described efforts by the FOLPROCOM consortium to exploit viral systems to tackle problems of protein folding, production and macromolecular assembly. There is considerable emphasis within the consortium on use of chaperones to promote folding *in vitro*, including the folding of proteins from inclusion bodies. Studies on bacteriophages and other viruses have led to the improvement in production of T4 fibre proteins assisted by T4 chaperone gp57, and co-expression of the gp31 chaperonin is predicted to improve further the yield and solubility of the T4 capsid protein. Production of viruses and subviral complexes has allowed structures of archael virus SH1, the *Thermus theromphilus* phage P23-77 and the dsRNA viruses phi6 and phi8 to be determined.

Fungal production of biopharmaceuticals is an attractive alternative to mammalian cells owing to system robustness, low cost and high product yields (up to 100 g protein l^{-1} has been reported). Peter Punt from TNO Quality of Life (Netherlands) presented the development of a low-protease activity *Chrysosporium* C1 strain (proprietary to Dyadic Intl.) suitable for production of full-length human antibodies. The protease-deficient mutants were selected as resistant variants on a medium containing a suicide substrate, while the glucoamylase carrier approach was used to achieve high levels of recombinant protein expression and secretion.

Reminding us that there is still no vaccine to protect against AIDS, Luca Vangelista (San Raffaele Scientific Institute, Milan) described efforts to find CCR5 antagonists that might be used in AIDS prevention. A major aim, the genetic engineering of RANTES to target the vaginal epithelium, has produced derivatives that are secreted from the Gram-positive host, *Lactobacillus jensenii*. The gene has been integrated into the lactobacillus chromosome, and the recombinant protein is secreted as a single form of the expected molecular mass. The purified protein still binds heparin and is fully active against HIV-1, indicating that it is functional. The Gram-positive theme continued with Jan Maarten van Dijl describing the exploitation of the secretion capacity of *Bacillus subtilis* for the production of difficult proteins. The major challenge to be overcome with this host is prevention of proteolysis, illustrated by the need to inactivate genes for eight extracellular proteases before interleukin 3 could be accumulated. The next task is to exploit the protein BdbD as a replacement for DsbA to achieve correct disulfide bond formation. Nicklas Bonander (Aston University, UK) described the use of the yeast *Saccharomyces cerevisiae* as a host for production of difficult proteins. One problem identified was a poor correlation between yield of mRNA and the accumulation of membrane proteins such as aquaporin. Considerable success had been achieved in improving protein yields by engineering the host, specifically deleting three genes that initially resulted in a 10-fold increase in RP yield, which was subsequently increased to 60-fold. These genes had been identified as target candidates from the transcriptomic study reported by the same group at the previous RPP meeting.

N-linked protein glycosylation: not only for eukaryotes!

E. coli remains the workhorse for over half of the recombinant proteins produced by the biopharmaceutical industries, but its Achilles heel is undoubtedly its inability to glycosylate eukaryotic proteins, which is not a trivial

problem given that two-thirds of lumen proteins are glycosylated! Coupled with the instability of mammalian cell lines and the density effect of the baculovirus system, this has been a major driver for the development of fungal expression hosts, culminating during the current meeting with the emergence of *Pichia pastoris* as the light at the end of the tunnel. However, the second plenary lecture by Marcus Aebi (ETH, Zurich) swept such preconceived ideas aside with his tour-de-force review of bacterial N-linked glycosylation systems. First discovered in *Campylobacter jejuni*, N-linked glycosylation genes are increasingly being recognised in a wider range of bacteria, especially in delta proteobacteria. The expression of these genes as a single operon offered the opportunity to teach *E. coli* to glycosylate RP. Key differences between the prokaryotic and eukaryotic systems include that the bacterial system does not require the target protein to be folded, as shown by glycosylation of folded proteins that are exported via the twin arginine targeting pathway. The bacterial lipid carrier differs from that of mammalian cells: bactoprenyl pyrophosphate instead of dolichol pyrophosphate; and the target site for glycosylation is slightly extended, there being a requirement for a glutamate or aspartate residue two amino acid N-terminal to the N-X-S/T sequence that is the glycosylation site in eukaryotes.

Genomic studies reveal vast differences in the oligosaccharide transferases across domains. In *Leishmania* there is a single component, but four variants of this protein that each can complement a glycosylation-deficient yeast. Each component confers different target specificity—but if *Leishmania* can cope with a single protein, why is there such a complex eight-component system in yeast?

Glycoengineering: from bacterial to mammalian cells

Five subsequent contributions selected from the offered papers included a summary of the risks and chances of protein glycosylation in different expression systems, as emphasised by Friedrich Altmann (Vienna) who warned that heterologous expression systems too often generate undesirable immunogenic structures. This can sometimes be avoided by propitious genetic engineering. Matthew DeLisa (Cornell, USA) extended the presentation by Marcus Aebi by summarising molecular tools available for studying bacterial N-linked glycosylation, stressing that prokaryote oligosaccharide transferases attach glycosyl residues to proteins after they have been exported into the periplasm—an analogy here between the eukaryotic Golgi body and the bacterial periplasm. The bottom line is that protein glycosylation and export are less tightly linked in bacteria than in mammalian cells. Mark Rendall (Lonza, UK) provided examples of the variability of CHO cells following transfection, a problem still not fully solved. Paolo Bartolini (San Paulo, Brazil) also emphasised the variety of glycosylated forms that can be generated in CHO cells, and again stressed that CHO cells are not yet a gold standard for the production of glycosylated proteins such as thyrotropin. Finally, Julian Ihssen (Empa, St. Gallen, Switzerland) presented process optimisation data for the high-level production of glycoconjugates in an *E. coli* fed-batch process. Favourable conditions were the use of glycerol as the energy-sufficient carbon source during the growth phase, and prolonged expression following induction at an OD_{600} of 30. This resulted in a 50-fold increase in yield relative to shake flask cultures. Glycosylation was the rate-limiting step.

Physiology, process development, process stability

Session six was opened by Gen Larsson (Stockholm, Sweden) who emphasised the effects that different feed profiles can have on RP productivity in *E. coli* during fed-batch fermentations. The successful approach reported involved exponential glucose feed that maintains a high growth rate (μ 0.5 h^{-1}) in addition to a very high specific productivity since a high intracellular translation capacity would be maintained. A low growth rate, on the contrary, was shown to give a lower synthesis rate but higher quality in terms of lower proteolysis and less inclusion body formation. Problems of acetate accumulation can be circumvented by switching to a constant feed rate, and thus the propagation to high cell densities is achieved. Since the fed-batch concept is very difficult to adopt in the high-throughput protein production format, for structural and functional determination of proteins, the development of engineered strains that do not accumulate acetate during fed-batch fermentations was also reported. This required deletion of genes for the PEP-phosphotransferase system to decrease the rate of glucose uptake and hence minimise acetate production. The levels of acetate produced were reduced to a minimum independently of the proteins investigated, namely, β-galactosidase and MalE31—the latter being a protein prone to forming inclusion bodies. The quality of MalE31 protein accumulated was also explored and was found to be dependent on very low IPTG induction concentrations and slow feed rates (and thus slow growth rate)—excellently complementing findings reported previously in the programme. Gerald Striedner (Vienna, Austria) described another strategy for increasing RPP by decreasing the burden on host cell metabolism. This involves integration of the target gene into the host chromosome. The transcription efficiency of the T7 polymerase compensates for the low gene dosage. Furthermore, expression rates can be fine tuned with the 'disposable cellular capacity' of the productive host, which would otherwise be overwhelmed by a high plasmid copy number and gene dosage in plasmid-based expression systems. High yields of several proteins were accumulated using both plasmid-free and plasmid-based *E. coli* expression systems.

Industrial scale protein production requires high cell density cultivation but the development of process intensification on a laboratory scale often provides unrealistic models of industrial scale production. Peter Neubauer (University of Oulu, Finland) described a scaled-down fed-batch technology, EnBase, that can be realised in a variety of formats (microplates, shake flasks) and can be a useful tool for high-throughput screening. In this strategy, the nutrient availability and hence the growth rate are controlled by slow release of glucose from starch via a unique regulating gel, by action of glucoamylase. In *E. coli* and *B. subtilis* expression systems, the EnBase technology produced up to 50 times more bacteria and up to 10 times more recombinant protein and more soluble protein than conventional microplate or shake flask cultures.

Silvana Becerra (University of Valparaiso, Chile) showed that by manipulating the environmental culture conditions (medium composition and cultivation temperature) during the proliferative stage of CHO cells cultivation, the yields of recombinant human tissue-type plasminogen activator in the second, productive, phase can be significantly increased. The proposed method of biphasic perfusion culture uses a slowly metabolised substrate, galactose, instead of glucose and decreased growth temperatures. However, the combination of factors that increased product yield when tested singly also increased cell productivity but dramatically decreased cell viability, indicating high stress levels associated with protein production and a requirement for further optimisation.

Chris Finnis (Novozymes Biopharma UK Ltd., UK) presented the development of baking yeast as an expression host for production of high

levels of biopharmaceutical recombinant transferrin, which is functionally equivalent to human serum transferrin. The development of the host has included multiple rounds of mutagenesis and screening for improved albumin secretion, followed by introduction of mutations in protease genes to overcome proteolysis, resulting in a 20-fold increase in productivity. In addition, the 2-micron plasmid was modified to express disulfide isomerase (ER chaperone) that increased cell productivity by a further 10-fold.

Post mortem of the meeting

So what were the four major take-home messages from this meeting? First, we still need a very wide range of hosts to produce recombinant proteins of quality adequate for use in human healthcare. CHO cells can produce excellent titres, but repeated reference was made to our inability to predict how a transfection line will perform, especially at high cell density when productivity declines. Second, there was the emergence of the *Pichia pastoris* system that potentially might replace mammalian cell cultures for many applications—but this remains a promise rather than a *fait accompli*! Third, a series of transcriptomic and proteomic studies generated large datasets and the conclusion that these databases will provide a valuable resource for understanding how to improve RPP. However, the reviewers are unable to cite many examples of how such data have already led to the design of a modified process resulting in higher yields of protein, the exception being the examples cited by Nic Bonander from Roslyn Bill's laboratory. Perhaps the most exciting outcome was the promise, which has almost been delivered, of bacterial hosts designed to yield correctly glycosylated mammalian proteins.

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