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Life Sciences and
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BioTech 2008 & 4th Swiss-Czech Symposium

Biopharmaceuticals: why use yeasts?

22–23 May 2008

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Campus Grüental, Wädenswil, Switzerland

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Biopharmaceuticals: why use yeasts?

Conference Proceedings of the BioTech 2008 and 4th Swiss-Czech Symposium held on 22 & 23 May 2008 in Wädenswil, Switzerland

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Table of contents

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The interplay between biology and industrial processes and the requisite knowledge of quality management in the area of pharma are becoming increasingly important.

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We are pleased to host BioTech 2008 and the 4th Swiss-Czech conference, which this year have been jointly organised under the umbrella of the recently founded Zurich University of Applied Sciences (ZHAW) and the 'new' Institute of Biotechnology (IBT) at the School of Life Sciences and Facility Management. On the initiative of the founding partners of AIBY (i.e. a cooperation network on Advances in Industrial Biotechnology of Yeasts), this bi-national event has now achieved international status. Moreover, the focus on enhanced yeasts as hosts for expressing therapeutic proteins, enzymes for the synthesis/modification of pharmaceuticals, drug target proteins, or proteins for diagnostics has attracted as many participants from industry as it has from academia. The conference is an excellent opportunity for knowledge sharing and transfer between these two groups.

Both internationalisation and close cooperation with industry are also the strategic directions of the recently established Master's degree programme in Pharmaceutical Biotechnology in Wädenswil. A sound Bachelor's programme (i.e. currently for about 150 students and costing around 6 mio CHF per annum), competent research with an annual turnover of about 2 mio CHF from third-party funding and R&D-networking through Biotechnet Switzerland are the pillars of the Institute of Biotechnology. PhD students from various foreign universities have been attracted to our institute, where they can take advantage of the unique equipment and know-how available to carry out sophisticated microbial or cell cultivations to complement their experiments.

As the interaction between biology and industrial processes and the requisite knowledge of quality management are increasing in importance, let us take this opportunity to jointly seek answers to the following questions and further develop our research and education programmes.

Which products can be made more effectively (and more safely) using enhanced yeast expression systems?

Which applications of yeasts lead to new properties and enhanced quality of products (i.e. biologically active molecules or bio-materials)?

How can yeast bioprocesses be made more predictable in order to facilitate process optimisation and strategic decision making?

We are sure you will enjoy your time in Wädenswil and that BioTech 2008 together with the 4th Swiss-Czech conference will add value to your business or research.

Our thanks go to all the numerous people who have helped in organising this event as well as to the event partners and advertisers for their much-appreciated support.

Prof Karin Kovar, Head of Bioprocess Technology

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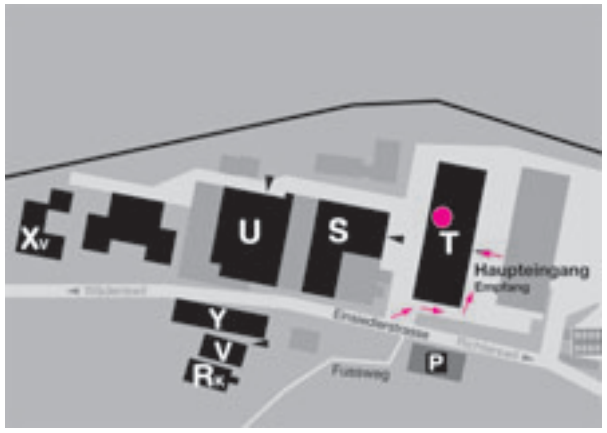

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Advertisements (listed in alphabetical order)

High-Level Protein Expression (Thursday 22 May 2008, Morning)		**page**
08:30 - 09:00	Registration	
Chairperson:	Anton Glieder	
Co-chairperson:	Verena Looser	
09:00 - 09:30	Opening session	
09:30 - 10:00	**A comparative view on yeast expression hosts *S. cerevisiae* and *P. pastoris*** Christine Lang, TU Berlin, Berlin D	17
10:00 - 10:10	Discussion	
10:10 - 10:40	Coffee break and exhibition & poster session	
10:40 - 11:10	**Fungal cell factories: production and screening of antibody (-fragments)** Peter J. Punt, TNO, AJ Zeist NL	18
11:10 - 11:20	Discussion	
11:20 - 11:50	**Intracellular expression of heterologous gene encoding bacterial penicillin acylase in *Pichia pastoris*** Pavel Kyslík, Institute of Microbiology ASCR, Prague CZ	19
11:50 - 12:00	Discussion	
12:00 - 12:40	**Genomics comes to the recombinant protein production yeast *Pichia pastoris*** James M. Cregg, Keck Graduate Institute of Applied Life Sciences, Claremont USA	20
12:40 - 12:50	Discussion	
12:50 - 14:00	Lunch & poster session and exhibition	

Process Engineering vs. Strain Design (Thursday 22 May 2008, Afternoon)		**page**
Chairperson:	Leona Paulová	
Co-chairperson:	Christian Meier	
14:00 - 14:30	**Alternative cultivation strategies with *Pichia pastoris* for the production of therapeutic recombinant proteins** Carmen Jungo, Lonza Ltd, Visp CH	21
14:30 - 14:40	Discussion	
14:40 - 15:10	**Production of cytochrome P450-metabolites using recombinant fission yeast *Schizosaccharomyces pombe*** Matthias Bureik, PomBioTech GmbH, Saarbrücken D	22
15:10 - 15:20	Discussion	
15:20 - 15:50	Coffee break and exhibition & poster session	
15:50 - 16:20	**Methanol free protein expression by *Pichia pastoris* using methanol inducible promoter variants** Claudia Ruth, Institute of Molecular Biotechnology, TU Graz A	23
16:20 - 16:30	Discussion	
16:30 - 17:00	**Strain design and its impact on process development in *H. polymorpha* and other yeasts** Gerd Gellissen, PharmedArtis GmbH, Aachen D	24
17:00 - 17:10	Discussion	
17:10 - 17:40	**Poster awards**	
17:40 - 19:00	Aperitif & poster session and exhibition	
19:00 - open end	Gala dinner	

Postranslational Modifications (Friday 23 May 2008, Morning)		**page**
08:30-09:00	Registration	
Chairperson:	Nico Callewaert	
Co-chairperson:	Sandra Núñez	
09:00 - 09:30	**N-linked protein glycosylation in yeast** Markus Aebi, Swiss Federal Institute of Technology (ETH), Zurich CH	25
09:30 - 09:40	Discussion	
09:40 - 10:10	**Secretory system engineered yeast expression systems: taking the prototypes further** Nico Callewaert, Flanders Institute for Biotechnology vzw, Ghent BE	26
10:10 - 10:20	Discussion	
10:20 - 10:50	Coffee break and exhibition & poster session	
10:50 - 11:20	***Yarrowia lipolytica:* a versatile expression system for the production of biopharmaceuticals** Adriana Botes, Oxyrane UK Ltd, Manchester UK	27
11:20 - 11:30	Discussion	
11:30 - 12:00	**Modified substrates in enzymatic glycosylations: a simple route to complex saccharides** Pavla Bojarová, Institute of Microbiology ASCR, Prague CZ	28
12:00 - 12:10	Discussion	
12:10 - 12:50	**Panel discussion**	
12:50 - 14:00	Lunch & poster session and exhibition	

Further Opportunities (Friday 23 May 2008, Afternoon)		**page**
Chairperson:	Hans-Peter Meyer	
Co-chairperson:	Radka Strnadová	
14:00 - 14:30	**Physiological characterization and strain engineering of *Pichia pastoris* by transcriptomics and proteomics** Brigitte Gasser, BOKU-University of Natural Resources and Applied Life Sciences, Vienna A	29
14:30 - 14:40	Discussion	
14:40 - 15:10	***Pichia pastoris*: a powerful expression system to support drug discovery projects in pharmaceutical industry** Ralf Thoma, F. Hoffmann-La Roche AG, Basel CH	30
15:10 - 15:20	Discussion	
15:20 - 15:50	Coffee break and exhibition	
15:50 - 16:20	**Enzymatic synthesis of glycoconjugates** Oreste Ghisalba, Novartis Pharma AG, Basel CH	31
16:20 - 16:30	Discussion	
16:30 - 17:00	**Co-expression of homologous and heterologous genes as tools for advancement of established and new yeast expression systems** Adelheid Degelmann, ARTES Biotechnology GmbH, Langenfeld D	32
17:00 - 17:10	Discussion	
17:10 – 17:40	**Complex mammalian proteins easily produced in *Pichia pastoris*: the case of vascular endothelial growth factors** Kurt Ballmer-Hofer, Paul Scherrer Institute, Villigen CH	33
17:40 - 17:50	Discussion	
19:00 - open end	Alumni get-together	

ORAL PRESENTATIONS

(abstracts listed in order of appearance in the conference programme)

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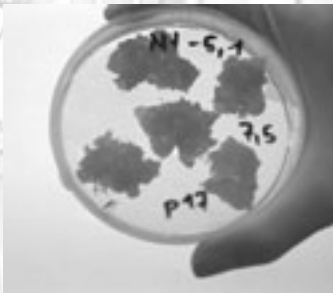

A comparative view on yeast expression hosts *S. cerevisiae* and *P. pastoris*

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Both the yeast *Saccharomyces cerevisiae* and *Pichia pastoris* are widely recognized and used as robust hosts for recombinant protein expression. We have established high throughput cloning and expression systems for both hosts and used them for comparative expression of almost 200 human cDNAs in structural genomics projects. Both yeasts proved to be reliable hosts giving overall expression success rates of 50 – 60 % of cDNAs tested. We have developed strategies to overcome bottlenecks in the production of sufficient amounts of soluble proteins by using different host systems in parallel. We further pursued the issue of understanding parameters that determine expression efficiency of proteins in yeast by reviewing expression behaviour and sequence-based parameters in about 70 homologous strains of *Pichia pastoris* harbouring different human cDNAs. More than ten sequence-based parameters with a possible influence on the expression level were analysed. Factors having a statistically significant association with the expression level were identified and include low abundance of AT-rich regions in the cDNA, isoelectric point of the recombinant protein and the occurrence of a protein homologue in yeast. Interestingly, some often discussed factors like codon usage or GC content did not show a significant impact on protein yield in our study. Further analysis of selected cDNAs revealed a correlation between mRNA structures and expression level.

Fungal cell factories: Production and screening of antibody (-fragments)

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Filamentous fungi are widely used for enzyme production for a wide variety of uses – for example food, feed, textile, paper and pulp, fuels and chemicals, detergents - due to the development of extremely productive strains and production processes. Levels in the range of 100 grams protein per liter have been reported, repeatedly. Therefore, these organisms have also been considered for the production of pharmaceutical proteins. In the pharmaceutical industry, the main production platforms are *E. coli* and mammalian cell lines. However, for those pharmaceutical proteins for which high yields and low production costs are important, filamentous fungi could provide a viable alternative.

We have explored the use of a highly productive fungal production platform (*Chrysosporium* C1) for the production of a very versatile class of pharmaceutical proteins, i.e., antibody molecules. Antibody molecules and molecules carrying antibody domains are currently the largest and fastest-growing class of biopharmaceuticals. Production of functional full-length human monoclonal antibodies has been accomplished using highly productive low protease mutant *Chrysosporium* host strains. High level expression was achieved using a glucoamylase-carrier approach, and recombinant strains expressing both heavy and light chains were obtained. Heterodimeric antibody molecules were formed efficiently, allowing simple purification of the protein from the culture fluid using protein A. Cell-based bio-assays performed on the culture supernatant and the purified samples revealed almost complete bioactivity.

Intracellular expression of gene encoding bacterial penicillin G acylase in *Pichia pastoris*

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Penicillin G acylase (PGA) is an important industrial enzyme that is used for the biotransformation of β-lactams. A processing of the inactive precursor of PGA to mature enzyme has been extensively studied for prokaryotic enzymes: the mature enzyme of *Escherichia coli* is a heterodimer synthesized as inactive preproprotein that contains the N-terminal leader sequence and proenzyme (proPGA) consisting of a smaller α- and bigger β-subunit separated by the spacer peptide. The first step of processing of preproprotein occurs in cytoplasm. Once still inactive precursor crosses the cytoplasmic membrane, resulting proenzyme is sequentially processed to form a mature enzyme.

A novel expression system based on the methylotrophic yeast *Pichia pastoris* X-33(pPIC-PA1) has been developed for PGA from *Escherichia coli* RE3: methanol-inducible expression of prokaryotic leader-less pga gene occurred in cytosol. The enzyme expressed in the yeast host (hPGA) was purified and characterized: in comparison with homologous PGA purified from the recombinant strain *Escherichia coli* RE3(pKA18), the activity of hPGA reached only 50% (32 U/mg of protein). Mapping of tryptic peptides of the mature enzyme expressed in yeast revealed different posttranslational processing of a proenzyme when compared to the process in bacterium. In addition to reduced activity of the enzyme, other characteristics of the mature hPGA were also affected.

Genomics comes to the recombinant protein production yeast *Pichia pastoris*

Cregg J.

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Pichia pastoris is a methylotrophic yeast that is in wide use for both basic and applied purposes. Its primary applied use is as a host system for the expression of foreign genes whose protein products are of interest to academic and industrial researchers. The expression system has been successfully used to produce over 1,000 foreign proteins, some at levels of greater than a gram/liter of culture. The genomic DNA sequence of an organism is an essential infrastructural prerequisite for entree into the modern field of genomics and to exploit the various “omics”-based approaches. In collaboration with Integrated Genomics (IG) of Chicago, IL and with the support of an STTR grant from the US Army Research Office to IG, a project to sequence the genome of *P. pastoris* was recently completed. A modified “shot-gun” sequencing strategy was pursued to generate a high-fidelity sequence. The current sequence has been assembled into 1544 contigs with an average length of ~6,100 bp and annotation efforts performed by IG using their proprietary software suite ERGO have identified ~6,000 genes. Searches of this database for 47 previously cloned and sequenced *P. pastoris* genes identified at least a major portion of each gene in our database. The primary future goal of this project is to develop a publicly accessible web site dedicated to the dissemination of information on the genome; and to construct and make available DNA microarrays focused on *P. pastoris* genes.

Alternative cultivation strategies with *Pichia pastoris* for the production of recombinant proteins

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The methylotrophic yeast *Pichia pastoris* is an attractive host for the expression of recombinant proteins. Advantages with this expression system include fast growth in inexpensive chemically defined media, expression linked to the strong and tightly regulated alcohol oxidase promoter *AOX1*, achievement of high cell densities and the possibility to secrete recombinant proteins to the culture medium.

Several therapeutic recombinant proteins have been produced at Lonza with this expression system up to a scale of 1500 L. After growth on glycerol to achieve high cell densities rapidly while repressing foreign gene expression, recombinant protein expression is usually induced by feeding methanol as sole carbon source.

As a result of the high heat of combustion of methanol, considerable heat is generated during the fed-batch stage on methanol. Therefore efficient cooling systems are required for high cell density *P. pastoris* processes. Moreover, since methanol is a highly reduced substrate, high oxygen transfer rates are required during the induction phase.

A detailed quantitative study on the use of mixed feeds of methanol and sorbitol in *P. pastoris* cultures has been performed. More precisely, the influence of the methanol-sorbitol ratio in the feed medium on growth stoichiometry and recombinant protein productivity was analysed in transient continuous cultures with linear changes of the methanol fraction in the feed medium. Results showed that use of mixed substrates can lead to increased volumetric productivity in recombinant protein and allow a significant reduction in heat production and oxygen consumption rates, features which are very advantageous in high cell density cultures, especially at large scale.

On the other hand, expression of recombinant proteins under the control of the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter constitutes an interesting alternative to the *AOX1* promoter because the use of methanol can be avoided. The expression of a therapeutic recombinant protein with these two promoters was compared.

Production of cytochrome P450-metabolites using recombinant fission yeast *Schizosaccharomyces pombe*

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[2] Department of Experimental and Clinical Toxicology, Saarland University, Germany

There is a growing demand for P450 metabolites of drug candidates by the pharmaceutical industry. In addition, P450 metabolites are continuously needed in clinical and forensic toxicology as new compounds appear in the illicit drug market. We and others have shown previously that the fission yeast *S. pombe* is a very well suited system for the expression of mammalian cytochromes P450. Therefore, it was the aim of this study to evaluate the feasibility of the synthesis of drug metabolites by biotransformation with recombinant fission yeast. For this purpose, fission yeast strains that express human liver P450 enzymes (CYP2B6, CYP2C9, CYP2C19, CYP2D6 & CYP3A4) were cloned and tested.

Conversion of several model drugs by biotransformation could be demonstrated at flask or fermenter scale. Identity and purity of the drug metabolites were tested by HPLC with ultraviolet (UV) detection, GC-MS, LC-MS or 1H-NMR. The time-space yield of biotransformations catalyzed by recombinant fission yeast strains was further characterized with respect to incubation pH, cell density, presence of glucose, gas exchange and incubation temperature on metabolite formation using a number of different substrates.

Biotransformation of drugs by recombinant fission yeast strains that express human liver P450 enzymes is shown. The drug metabolites could be obtained in sufficient quantities to allow their comprehensive chemical characterization. These results allow us to provide industry and academia with a fast and convenient access to P450 metabolites.

Methanol free protein expression using methanol inducible promoters in *Pichia pastoris*

Ruth C.[1], T. Zuellig[2], A. Mellitzer[1], F. S. Hartner[3], V. Looser[2], D. Langenegger[2], P. Hyka[2], K. Kovar[2] and A. Glieder[1]

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During the last decade, the methylotrophic yeast, *Pichia pastoris*, has become a major eukaryotic host for recombinant protein production (Cereghino *et al*., 2000). Expression of hundreds of different proteins has been reported so far. Several processes, employing *P. pastoris* expressed biocatalysts (enzymes), were implemented on industrial scale (Hasslacher *et al*. 1997; Glieder *et al*. 2003). One major reason for the success of this yeast is the inducible alcohol oxidase I (*AOX1*) promoter, which is tightly repressed in presence of glucose, glycerol and other carbon sources and strongly induced by methanol in absence of repressing carbon sources (Cereghino *et al*., 2000). However methanol is toxic, flammable and its presence in growth media might also be unfavourable for some sensitive secreted products (*e.g.* biopharmaceuticals).

Based on sequence analyses, we modified the *AOX1* promoter to generate promoter variants with altered expression levels and regulatory properties. Finally we created a synthetic promoter library, in order to facilitate the identification of the perfectly matching promoter/target gene combination. Several reporter proteins like the diagnostic reporter HRP (Horseradish peroxidase), the industrial enzyme CalB (*Candida antarctica* lipase B) and porcine Trypsinogen, which is used in processing of biopharmaceuticals, were expressed using the modified promoter variants. The properties of the generated libraries were studied and resulted in some promoters with more efficient expression in *Pichia pastoris* and some new promoter variants with high expression upon glucose depletion, providing the opportunity of methanol free protein expression using a methanol inducible promoter (Weis *et al*. 2004; Morawski *et al*. 2000, Hanquier *et al*. 2003, Rotticci-Mulder *et al*. 2001).

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Strain design and its impact on process development in *H. polymorpha* and other yeasts

Merckelbach A., G. Hehmann, G. Melmer and G. Gellissen

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Yeasts are excellent producers of recombinant proteins, especially pharmaceuticals, and they include a great diversity of organisms. Some yeast platforms like *Hansenula polymorpha* are distinguished by a growing track record as producers of proteins that have already reached the market whereas other newly defined systems like *Arxula adeninivorans* have yet to establish themselves (Gellissen *et al*., 2004). Accordingly, a range of defined industrial *H. polymorpha*-based processes exist and can be re-assessed for strain and process design. Most of the established industrial processes lean on the use of promoters derived from *MOX* and *FMD*, genes of the methanol metabolism pathway. These genes are de-repressed upon depletion of a range of carbon sources as recently confirmed by transcriptome analysis, in contrast to a strictly methanol-dependent induction of such promoters in other methylotrophic yeasts. Due to these characteristics fermentation modes can be defined for recombinant strains harbouring such expression control elements that lean on a limited supplementation of glycerol or glucose to a culture medium (Kang *et al*., 2005). Use of other promoters requires different regimens of carbon and nitrogen supplementation. For fermentation of *H. polymorpha* a synthetic medium (Syn6) has been developed. Depending on strain design suitable pH conditions have to be assessed and to be defined.

No industrial process has been developed so far for *Arxula adeninivorans* and only a limited range of strong promoter elements exists, suitable for heterologous genes expression. First examples indicate that Syn6 originally designed for *H. polymorpha* provides a suitable fermentation medium for this dimorphic yeast. Characteristics like osmo- and thermotolerance can be addressed for the definition of culture conditions.

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N-linked protein glycosylation in yeast

Aebi M.

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N-linked protein glycosylation is the most frequent protein modification in eukaryotic cells. This process initiates at the membrane of the Endoplasmic Reticulum, where an oligosaccharide, $Man_5GlcNAc_2$, is assembled on the lipid carrier, dolichylpyrophosphate, translocated across the membrane and completed to $Glc_3Man_9GlcNAc_2$. This oligosaccharide is then transferred to selected asparagine residues of nascent polypeptide chains.

N-linked protein glycosylation does also take place in archaea and in bacteria. The recently discovered N-linked protein glycosylation process in *Campylobacter jejuni* was transferred into *Escherichia coli*, enabling a genetic and biochemical analysis of the prokaryotic pathway. The high sequence similarity of the bacterial oligosaccharyltransferase with one subunit of the eukaryotic enzyme, the very similar protein acceptor sequence as well as the finding that oligosaccharides linked to isoprenoid lipids serve as substrates in the reactions suggest that the bacterial and the eukaryotic N-linked protein glycosylation are homologous processes. In contrast to the bacterial process, N-linked glycosylation in eukaryotes occurs before folding of the protein, enabling the use of the N-linked glycan as a general signal that reflects the folding status of the protein. The direct comparison of the homologous process in pro- and eukaryotes made it possible to formulate hypotheses regarding the eukaryote-specific components of the pathway. In particular, the role of eukaryote-specific subunits of the oligosaccharyltransferase complex can be addressed.

Secretory system engineered yeast expression systems: taking the prototypes further

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Over the past several years, we and others have developed *Pichia pastoris* yeast strains which are capable of modifying recombinant proteins produced in them with human-type bi-antennary galactosylated N-glycans. However valuable these proof-of-concept studies have been, they are not the end of the story in this field: it is still a significant challenge to achieve engineering efficiencies that are robust enough to yield >90% of the desired human glycoform of a broad range of proteins under different fermentation regimens. In this presentation, insight will be given in the workflows that are used to come closer to this goal and thus to true biotechnological application of these engineered expression systems.

Yarrowia lipolytica: a versatile expression system for the production of biopharmaceuticals

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Y. lipolytica has several unique characteristics that makes it highly suitable for the functional expression of both secreted and membrane proteins. A range of newly developed tools for this expression system now allows its exploitation for a wide range of applications including whole cell biocatalysis, therapeutic glycoprotein production, antibody engineering and drug discovery.

The innate ability of this yeast to utilise hydrophobic substrates allowed the development of highly efficient whole cell biocatalytic processes for key steps in the synthesis of chiral API's. In whole cell biocatalysis, one of the major constraints is low volumetric productivity due to the low water solubility of substrates. To overcome mass-transfer limitations, most industrial biocatalytic processes require the addition of water-immiscible solvents as holding phases for substrates or the addition of co-solvents to increase the water solubility of the substrates. We developed two-phase biocatalytic processes for the hydrolysis of highly hydrophobic and even solid epoxides without the addition of solvents or co-solvents.

Efficient secretion of heterologous proteins by the production host is essential for the manufacturing of therapeutic proteins. To validate the secretory capability of strains developed for the production of therapeutic proteins, we employed an enzyme with two glycosylation sites as model protein for process development. Unoptimised production of the enzyme during fed-batch cultivation of the production strain in defined media exceeded 2 g/l and comprised > 80% of total secreted protein.

Control over *N*-glycosylation is a key requirement for the production of therapeutic glycoproteins. The unique features of the glycosylation pathway of this host allowed the development of novel engineering strategies to construct strains for the production of secreted glycoproteins with unrivalled homogeneity. These strains can now be employed for the production of glycoproteins with homogeneous $Man_8GlcNAc_2$, $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$ *N*-glycans. The development of strains that will allow the production of glycoproteins targeted to mannose 6-phosphate receptors is in progress. The complete absence of fucose from the *N*-glycans has potential application for the production of therapeutic IgG antibodies with enhanced ADCC.

The superior capability of the host to produce integral membrane proteins, achieving *N*-glycan homogeneity, the recent development of tools to expand the membrane capacity and to tightly regulate expression of heterologous genes now allows the investigation of the ability of this host to produce transmembrane proteins such as GPCR's.

To further expand the utility of this expression platform, we have successfully developed the tools required to utilise this expression system to generate libraries of proteins displayed on the cell surface. This novel platform provides an alternative yeast surface display platform for protein engineering and characterisation in a host with proven ability to functionally express and secrete complex eukaryotic proteins that are often difficult to produce in other hosts.

Modified substrates in enzymatic glycosylations: a simple route to complex saccharides

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Filamentous fungi are an abundant source of extracellularly produced glycosidases (Bojarová-Fialová *et al*., 2007). These enzymes are stable, readily available and stereoselective. Their broad substrate specificity enables one-step synthesis of oligosaccharides using donors and acceptors with desired structural modifications. Molecular modelling is a convenient tool for predicting the binding of the substrate to the enzyme active site, which is helpful for the design of good substrates and inhibitors. The yields of transglycosylation reactions commonly range between 20-40%; however, they may even exceed 75%. Modified carbohydrate donors and acceptors are prepared by standard chemical procedures or by a combination with another enzyme system, *e. g.*, galactose oxidase, laccase or lipase/protease. The enzymes can readily be used as crude $(NH_4)_2SO_4$ precipitates of the induced medium that contain minimum interfering side activities. A heterologous expression of glycosidases in various systems including yeast is very challenging; *i.a.* due to the necessity of co-expressing a chaperone and the heavy glycosylation of these eukaryotic enzymes (Plíhal *et al*., 2007).

A good example of a combination of *in vivo* and *in vitro* approaches is the synthesis of a strong trisaccharide ligand to CD69 activation receptor of natural killer cells, δ-D-GalNAcA-(1-4)-δ-D-GlcNAc-(1-4)-D-ManNAc (Bojarová *et al*., 2008). This compound was prepared in a transglycosylation reaction catalysed by δ-*N*-acetylhexosaminidase from *Talaromyces flavus*, followed by *in situ* oxidation by $NaClO_2$. The donor *p*-nitrophenyl-2-acetamido-2-deoxy-δ-D-*galacto*-hexodialdo-1,5-pyranoside originated by the action of galactose oxidase.

Glycosyl azides, which have recently been designed as novel glycosidase donors (Bojarová *et al*., 2007), offer a worthwhile possibility of coupling to stabilizing scaffolds through click chemistry or amidation to yield immunoactive glyconjugates.

Thus, by a fusion of a careful selection of strain and cultivation conditions and control of the transglycosylation process, we may efficiently and selectively produce oligosaccharides that carry target structural modifications. Through chemical post-treatment, these structures can easily be further modified or coupled to complex glycomimetics, applicable for example in medicine (activation of natural killer cells). Although the enzymatic synthesis is not a panacea for all synthetic problems, it represents an elegant green solution to the vicious circle of tendious protection and deprotection steps needed in organic chemistry.

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Physiological characterisation and strain engineering of *Pichia pastoris* by transcriptomics and proteomics

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(Genome annotation, microarray development, and the application of –omics to analyze cellular responses to protein overexpression and fermentation conditions, and the identification of secretion enhancers).

To accelerate the development of production strains it is crucial to understand the molecular physiology of the host, and the specific limitations that the product may exert on expression. However, for many industrially important microorganisms the lack of commercially available microarrays still hampers physiological research on a genome wide level. Exemplarily, the background of protein folding and secretion in the yeast *Pichia pastoris* is presently widely dependent on conclusions drawn from analogies to *Saccharomyces cerevisiae*. To close this gap for a yeast species employed for its high capacity to produce heterologous proteins, we developed full genome DNA microarrays for *P. pastoris*, aiming at their application for studying host physiology. To evaluate the suitability of the microarray, we analyzed the unfolded protein response (UPR) in *P. pastoris*, as compared to *S. cerevisiae*. The differences observed between *P. pastoris* and *S. cerevisiae* underline the importance of DNA microarrays for industrial production strains. Overexpression of the UPR transcription factor *HAC1*, the most direct control for UPR genes, resulted in significant new understanding of this important regulatory pathway in *P. pastoris*, and generally in yeasts. Recently, we have successfully proven the value of transcriptomics for the targeted improvement of the *P. pastoris* protein production platform. Screening the whole genome for factors involved in protein secretion, and further in silico selection lead to the identification of novel secretion enhancers with a 60% success rate. Both specific production rates as well as volumetric productivity of an antibody fragment could be improved 2.5-fold by this approach (Gasser et al., 2007a). The new microarrays will be applied in the near future to understand in detail why low temperature (Gasser et al., 2007b), and low oxygen supply (Baumann et al., 2007) enhance protein secretion in *P. pastoris*. Additionally, 2D difference gel electrophoresis (2D-DIGE) and subsequent identification of *P. pastoris* proteins by LC-ESI-MS2 has been established in our lab. Taken together, these global transcriptomic and proteomic approaches should lead to a better understanding of host cell physiology and to decipher additional physiological bottlenecks impeding heterologous protein production in *P. pastoris*.

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Pichia pastoris: a powerful expression system to support drug discovery projects in pharmaceutical industry

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For enabling investigation of drug targets by a biostructural approach in pharmaceutical industry the time frame is quiet short to have an impact on lead identification or optimization in the drug discovery process. In such a context a parallel approach is the only way to be successful. A wide range of different expression systems (e.g. *E. coli*, *Pichia pastor*is, *Baclo*-virus and mammalian cells) and vectors are used to set up an efficient protein production line. In addition to this narrow timelines we are facing over the last years a shift to drug targets very difficult to express like membrane proteins or multidomain proteins. Based on the experience of such an approach *Pichia pastoris* offers some advantages for biostructural work. On two important drug targets a secreted protease (Dipeptidypeptidase IV) and a membrane protein (Oxido-squalene cyclase) I would like to exemplify the high impact of such an expression system in the drug discovery process.

Enzymatic synthesis of glycoconjugates

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Complex carbohydrates and glycoconjugates are crucial in fertilisation, embryogenesis, neuronal development, and cellular proliferation processes as well as being used as blood group determinants and for the secretion and circulation half-life of glycoproteins. For the manufacturing of carbohydrate-based pharmaceuticals, both high structural complexity and sophisticated synthesis strategies are required. Although such strategies are available from academic research, their practical realisation on an industrial scale is still hampered by many economical and technological limitations.

One of the most typical reactions for the formation of C-C bonds is aldolic condensation. In nature, such stereo-controlled reactions are catalyzed by enzymes classified as lyases (EC 4). The majority of these enzymes can be found in the biosynthesis of carbohydrates, and are used for the synthesis of natural and unnatural carbohydrates. Aldolases accept a wide range of aldehydes in place of their natural substrates and permit the synthesis of carbohydrates such as aza-, deoxy-, deoxythio-, fluoro-sugars and C8 or C9 sugars.

The introduction of biotechnology into carbohydrate chemistry, with its advantages of high selectivity, specificity, and the catalytic power of enzymes to form glycosidic bonds, enables new straightforward synthesis routes. The sugar nucleotide-dependent glycosyltransferases have proved to be the most suitable for the synthesis of complex oligosaccharides, as these enzymes catalyze stereo- and regiospecific reactions (high specificity and high yields) with a broad range of complex acceptor structures. Of interest for the *in vitro* synthesis of glycoconjugates and glycans are the following families: fucosyl-, galactosyl-, mannosyl, N-acetylgalactosaminyl-, N-acetylglucosaminyl-, and sialyltransferases. All of the essential human glycosyltransferases are nowadays available as affordable recombinant enzymes, e.g. produced with *Pichia pastoris* expression systems. The all-important prerequisite for the future industrial development of carbohydrate chemistry and glycotechnology remains the establishment of economically affordable access to bulk quantities of all necessary types of (recombinant) glycosyltranferases and sugar nucleotides. If this can be achieved, glycosyltransferase-mediated glycosylation coupled with *in situ* regeneration of sugar nucleotides will become the most effective method for large-scale stereo-controlled synthesis of oligosaccharides and glycoconjugates.

For instance, a new industrial avenue to oligosaccharide synthesis was opened by an approach using whole-cell biotransformations. Researchers from Kyowa Hakko Co. (Japan) developed systems for the large-scale production of UDP-Gal and globotriose from inexpensive starting materials by coupling metabolically engineered bacteria. The production system for UDP-Gal combines a metabolically engineered *E. coli* NM522/pNT25/pNT32 with *Corynebacterium ammoniagenes* DN510 producing uridine 5'-triphosphate (UTP). This system is reported to accumulate 44 g l-1 (72 mM) UDP-Gal after 21 h of reaction.

The strategy of producing sugar nucleotides by combining metabolically engineered *E. coli* with a nucleoside 5'-triphosphate-producing micro-organism and the concept of producing oligo-saccharides by coupling such systems with glycosyltransferases have a great potential for application in the manufacture of other sugar nucleotides and oligosaccharides, and may even be used for other types of glycoconjugates in the future.

Co-expression of homologous and heterologous genes as tools for advancement of established and new yeast expression systems

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The methylotrophic yeast *Hansenula polymorpha* has proven to be a robust and cost-effective platform for the production of many types of pharmaceutically interesting proteins and technical enzymes. Heterologous expression of industrially valuable proteins on a large scale, however, sometimes requires additional improvements beyond the use of strong promoters and optimization of gene dosage, if production of the target protein is to be commercially viable. Co-expression of chaperones, foldases, or other facilitators of secretion is one way to enhance production rates in various expression systems. In *H. polymorpha*, this strategy has resulted in enhanced secretion efficiency of a number of structurally and functionally diverse proteins. Examples of the co-overexpression strategy to increase production rates in *H. polymorpha* will be described. A promising and versatile expression platform based on the dimorphic yeast *Arxula adeninivorans* will also be introduced. In this system, co-expression of several heterologous genes has been employed successfully to enhance performance in metabolic engineering and biocatalysis applications.

Complex mammalian proteins easily produced in *Pichia pastoris*; the case of vascular endothelial growth factors

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The development of functional blood and lymphatic vessels requires spatio-temporal coordination of the production and release of growth factors such as Vascular Endothelial Growth Factors (VEGFs) by stromal and hematopoietic cells. VEGFs are encoded by several genes giving rise to VEGF-A, -B, -C, -D, -E, -F and PlGF. All VEGFs are cysteine crosslinked dimers that bind to three tyrosine kinase receptors, VEGF receptor 1-3, which are predominantly expressed on endothelial and hematopoietic cells. Each VEGF subfamily gives rise to additional isoforms generated upon splicing of the primary transcript. VEGFs assume a highly complex tertiary structure requiring proper formation of several cysteine crosslinks giving rise to a so called cysteine knot structure. Production of VEGF in bacteria has been notoriously difficult since the material, as a consequence of the lack of glycosylation and because cysteine crosslinks are not properly formed, end up in inclusion bodies. Refolding from denatured bacterially produced protein is inefficient and tedious. We chose *Pichia pastoris* to express VEGF proteins and have now demonstrated the large scale production of more than 20 VEGF variants in this microorganism in biologically active form. To improve reproducibility in large scale production we use a modified version of a fermenter marketed for bacterial production of proteins.

We recently obtained a crystal structure of a viral VEGF isoform which shows the competence of *Pichia pastoris* in the production of such highly complex mammalian proteins. We also obtained an electron microscopic structure of a ligand/receptor complex using mammalian and yeast produced proteins. VEGFs made in *Pichia pastoris* are highly stable over weeks and maintain biological activity as shown in assays such as collagen invasion of endothelial cells, induction of angiogenesis on the chicken chorioallantois or *in vivo* angiogenesis induced by VEGF implants in a mouse model system.

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POSTER PRESENTATIONS

(listed according to the programme sessions

- in alphabetical order of the first author's name

- underlined: names of the participants attending the BioTech 2008)

GMP biomanufacturing | GMP aseptic filling & lyophilisation | GMP cell encapsulation

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PART I

1. New *Schizosaccharomyces pombe* based system to discover industrial enzymes
Andryushkova A., C. Gruber, S. Abad and A. Glieder

2. Hypoxic fedbatch cultivation of *P. pastoris* increases productivity of recombinant proteins
Baumann K., M. Maurer, M. Dragosits, O. Cos, P. Ferrer and D. Mattanovich

3. Xplor Yeast Expression System - find the optimal host to overexpress your gene
Böer E., A. Degelmann, G. Steinborn, M. Piontek and G. Kunze

4. Generation of microbial strains for production of therapeutic human peptide hormone
Chrastilová Z., M. Hladíková, A. Dziahtsiaryk, M. Macková, V. Král and J. Ludwig

5. Efficient protein expression in *Pichia pastoris* using methanol and glucose simultaneously
Hyka P., D.S. Langenegger, V. Looser, S. Núñez, K. Melzoch, H.-P. Meyer and K. Kovar

6. Expression of four different laccases from *Trametes versicolor* in *Pichia pastoris*
Koschorreck K., S. Richter, R. Schmid and V. Urlacher

7. Use of site-specific recombination to create a *Pichia pastoris* Mut^{s} strain
Näätsaari L. H., C. Ruth and A. Glieder

8. A kit for fast and efficient expression and directed evolution of proteins in *Pichia pastoris*
Pscheidt B.[1], Z. Liu[1], C. Ruth[2] and A. Glieder[1,2]

9. c-LEcta's *Pichia* expression platform for the screening and production of enzymes
Schmiedel R. and A. Vogel

10. Novel promoters for protein production in *Pichia pastoris*
Stadlmayr G., B. Gasser, M. Rothmüller, M. Sauer, and D. Mattanovich

PART II

11. Vitality of *Pichia pastoris* yeast during heterologous protein production phase
Branská B., L. Paulová, M. Linhová and K. Melzoch

12. Recombinant protein production in *Pichia pastoris* using byproduct of biodiesel production
Çelik E., N. Ozbay, N. Oktar and P. Çalık

13. Design of a production process for the plant enzyme XET in *Pichia pastoris*
Henriksson M., M. Jahic, H. Brumer, S.O. Enfors and T. Teeri

14. Determination of physiological changes during fedbatch processes with *P. pastoris* expressing recombinant proteins
Hyka P., S. Núñez, V. Looser, B. Philip, K. Melzoch and K. Kovar

15. Screening and production of *Candida antarctica* lipase B in *Pichia pastoris*; inducible versus constitutive expression
Larsen, M. W., M. Jahic, S-O. Enfors and K. Hult

16. Production of recombinant protein and physiology of *Pichia pastoris* Mut^{+} strain grown in continuous culture
Paulová L., B. Branská, S. Vilčková and K. Melzoch

17. High throughput screening for factors enhancing protein production and secretion with *Pichia pastoris*
Stadlmayr G., K. Benakovitsch, B. Gasser, M. Sauer and D. Mattanovich

18. Citric acid production from sucrose by *Yarrowia lipolytica* recombinant strains
Walczak E., Z. Lazar and M. Robak

PART III

19. Recombinant human growth hormone production in *Pichia pastoris*
Çalık P., M. A. Orman, E. Çelik, M. Halloran, G. Çalık and T. H. Özdamar

20. Alpha-L-Fucosidase and beta-D-fucosidase activities of *Paenibacillus thiaminolyticus*
Benešová E.[1], P. Lipovová[1], J. Riedl[1] and B. Králová[1]

21. Engineering of the *Yarrowia lipolytica* protein expression system
De Pourcq K.[1], A. Van Hecke[1], S. Geysens[1], W. Vervecken[1], A. Botes[3] and N. Callewaert[1,2]

22. Characterization of the *HAC1* gene from *P. pastoris* and evaluation of the effect of its overexpression on the secretion of heterologous proteins
Guerfal M., S. Ryckaert, P. Jacobs, R. Derycke and N. Callewaert

23. Production of humanized therapeutic proteins in the yeast *Pichia pastoris*
Jacobs P., S. Geysens, W. Vervecken, V. Kaigorodov, R. Contreras and N. Callewaert

24. Cell wall-anchored mannoprotein variants engineered to enhance metallosorption by *Saccharomyces cerevisiae*
Kotrba P., J. Káš and T. Ruml

25. Production of recombinant mGM-CSF in glycoengineered *P. pastoris*
Meier C.[1,2], S. Núñez[1], P. Hyka[1], P. Jacobs[2], N. Callewaert[2] and K. Kovar[1]

PART IV

26. Fast gene copy number determination in the methylotrophic yeast, *Pichia pastoris*
Abad S.[1], K. Kitz[1], F. Hartner[1] and A. Glieder[2]

27. Process development for the production of 3-DMC- an anti-cancer drug at commercial scale
Dubey K. and B.K. Behera

28. Establishment of a novel system for the detection of spoilage microorganisms in wine
Drissner D., A. Bachmann, A. Frei, N. A. Porret, K. Schneider, F. Hesford and J. Gafner

29. The method of image analysis as new tool for monitoring of *Pichia* cell aggregation
Krulikovská T., P. Patáková, J. Heřman and O. Schreiberová

30. Engineering of plant vacuolar proteins for production in *Pichia pastoris*
Kowalska M., P. Galuszka and I. Frébort

1. New *Schizosaccharomyces pombe* based system to discover industrial enzymes

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Biotechnological approaches to obtain substances, previously not available or available using costly chemical synthesis, are constantly growing in importance. DNA from the huge variety of organisms and environmental samples was used in screening programs for new interesting enzymes, creating such substances. But traditional screening systems are based on prokaryotic expression hosts (e.g. *E. coli*). This dramatically limits the spectrum of eukaryotic proteins expressed in active form. As alternative screening host *S. pombe* is unique: 1) unicellular (easy handling), 2) high molecular similarity to higher eukaryotes compared to other yeasts (complex posttranslational modifications), 3) high transformation rates (10^6/µg DNA) allow production of representative DNA libraries. Being also able to express biologically functional protein from unmodified cDNA sequences from different higher eukaryotic sources *S. pombe* can be conveniently used for screening DNA libraries from such organisms. But till now commercially available systems were lacking small vectors suitable for efficient cloning of big inserts, which is crucial for construction of representative DNA libraries especially if genomic DNA is used. Thus a series of vectors of less than 4 kb with strong inducible or constitutive promoters was constructed. Convenient selection based on antibiotic resistance is possible and allows to use robust and fast growing wild type strains as hosts. With this new expression system, together with our new solid phase high throughput screening methods for *S. pombe*, screening for new perspective enzymes with biocatalytic application in industry and pharmacy will be greatly facilitated.

2. Hypoxic fedbatch cultivation of *P. pastoris* increases productivity of recombinant proteins

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High cell density cultivation of *Pichia pastoris* has to cope with several technical limitations, most importantly the transfer of oxygen. By applying hypoxic conditions to chemostat cultivations of *P. pastoris* expressing an antibody Fab fragment under the *GAP* promoter, a 2.5 fold increase of the specific productivity q_P at low oxygen supply was observed. At the same time the biomass decreased and ethanol was produced, indicating a shift from oxidative to oxido-fermentative conditions. Based on these results we designed a feedback control for enhanced productivity in fed batch processes, where the concentration of ethanol in the culture was kept constant at approximately 1.0% (v/v) by a regulated addition of feed medium. This strategy was tested successfully with three different protein producing strains, leading to a 3 to 6 fold increase of the q_P and 3 fold reduced fed batch times. Taken together the volumetric productivity Q_P increased 2.3 fold.

K.B. and M.M. contributed equally to this work.

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3. Xplor Yeast Expression System - find the optimal host to overexpress your gene

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For several protein targets the initial step towards commercial manufacturing is the careful and correct choice of the best suited expression system. The Xplor Vector System, developed at the IPK Gatersleben and provided by ARTES, is an ideal tool to select the optimal host from available yeast platforms. While quantitative aspects, *e.g.* the application of strong and inducible promoter elements, are addressed at a later stage, expression using the Xplor Vector System answers relevant quality issues such as processing, stability, and post-translational modification (Terentiev *et al*., 2004).

The Xplor Vector System is composed of a fixed backbone for propagation in *E. coli* and individual modules for: integration into the yeast's rDNA, selection of integrants (auxotrophic and dominant markers), transient or permanent autonomous replication and recombinant gene expression cassette (constitutive, wide range promoter and terminator) (Steinborn *et al*., 2005 and 2007a; Böer *et al*., 2005).

From the resulting vector portfolio expression cassettes can be excised for single or multi-copy integration applying strong or weak selection marker, respectively. Furthermore, the expression cassettes can be designed in such a way that the generated integrants lack any bacterial sequences (Steinborn *et al*., 2007b).

The expression cassettes are stably integrated into the rDNA of yeast genomes. The approach of comparative expression from a single vector source is designed for virtually any yeast platform and verified up to now for: *Arxula adeninivorans* (budding and filamentous cells), *Hansenula polymorpha, Saccharomyces cerevisiae, Pichia pastoris, Yarrowia lipolytica*, *Debaryomyces polymorphus* and *hansenii* (Terentiev *et al*., 2004; Böer *et al.*, 2007).

Wild type and auxotrophic yeast strains were transformed with wide range vectors containing expression cassettes for IL6 (interleukin 6), phytase and interferon α. For the first two recombinant proteins host-specific differences in expression level and in posttranslational modifications were observed (Böer *et al*., 2007), interferon α is currently being under examination.

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Böer, E., Steinborn, G., Matros, A., Mock, H.P., Gellissen, G. and Kunze, G. (2007) Production of interleukin-6 in *Arxula adeninivorans*, *Hansenula polymorpha* and *Saccharomyces cerevisiae* by applying the wide-range yeast vector (CoMed) system to simultaneous comparative assessment. FEMS Yeast Res. 7, 1181-1187.

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Steinborn, G., Gellissen, G. and Kunze, G. (2007b) A novel vector element providing multicopy vector integration in *Arxula adeninivorans*. FEMS Yeast Res. 7, 1197-1205.

Terentiev, Y., Pico, A.H., Böer, E., Wartmann, T., Klabunde, J., Breuer, U., Babel, W., Suckow, M., Gellissen, G. and Kunze, G. (2004) A wide-range integrative yeast expression vector system based on *Arxula adeninivorans*-derived elements. J. Ind. Biotechnol. 31, 223-228.

4. Generation of microbial strains for production of therapeutic human peptide hormone

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Therapeutically active peptides and proteins (biopharmaceuticals or biodrugs) represent a rapidly growing proportion of marketed drugs and have an undisputed place alongside many therapies; for certain indications they even are the only effective therapy. Therapeutic peptides and proteins can (in some cases) efficiently affect targets that do not bind small chemistry-based molecules with sufficient affinity. Biopharmaceuticals cover many therapeutic areas including treatment of cancer, autoimmune diseases, diabetes, anemia, disorders associated with lack of certain proteins (e.g. human growth hormone) and others. Originally, therapeutic peptides and proteins were extracted from natural sources but then their production has shifted to new and more advantageous biotechnological approaches such as recombinant DNA and hybridoma technologies (for production of monoclonal antibodies). These new techniques also allow engineering of peptides and proteins for optimal pharmacological properties. Different types of expression hosts are used or production of biopharmaceuticals: bacteria, yeast, insect, mammalian and plant cells. Recently also transgenic plants and animals have been explored for their ability to produce therapeutically active peptides and proteins. The aim of this work is to generate a high-level expression system for production of recombinant human peptide which can be used for pharmaceutical purposes. The production of biologically active peptides in bacteria (*Escherichia coli*) unfortunately has met limited success due to the low yield, presumably related to the rapid intracellular degradation of the peptides, as well as the difficulty in purification from contaminating proteins and peptides. Several methods leading to increased yield can be used. One method relies on the use of fusion partners (glutathione S-transferase (GST), maltose binding protein (MBP and others). By including an appropriate protease recognition sequence, the peptide can be separated from the fusion partner by proteolytic cleavage. Another method involves gene polymerization. Here, the gene of interest is expressed and purified as polymer and subsequently cleaved into monomers. A third approach is to express the target gene in a bacterial strain exhibiting low proteolytic activity what should also lead to higher yields of the produced peptide. We have developed several bacterial expression systems, using all three approaches mentioned for yield enhancement. As an alternative to bacterial expression, we are also developing a yeast expression system, producing and secreting the desired peptide. The main goal of our work is to evaluate several expression systems suitable for production of short peptides (bacterial and yeast) and to identify the best method for high-level expression at low cost.

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5. Efficient protein expression in *Pichia pastoris* using methanol and glucose simultaneously

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A process strategy for the manufacture of heterologous proteins in *Pichia pastoris* Mut$^+$ using glucose alongside methanol has been developed and tested with different products (*e.g.* trypsinogen, horseradish peroxidase, beta-galactosidase, mGM-CSF). In contrast to the most widespread technology (*i.e.* consisting of biomass growth with glycerol and a subsequent production phase with pure methanol; Invitrogen, 2002), higher rates of product formation could be achieved with this method than by using methanol alone. As a result, (i) the process duration is significantly shorter, (ii) the heat evolved is reduced, and (i) process robustness is enhanced.

As a paradigm, data on porcine trypsinogen expressed in HCD (high-cell-density) fedbatch-cultures at pH 6 and 30 °C are presented. The cultures were either fed with a mixture of glucose and methanol (40%:60%, ratio of carbon in the mixture) or with only 100% methanol. Both of these cultures were comparable with respect to biomass growth and product expression as determined by the specific (enzyme) activity. Thus, glucose did not negatively affect (*i.e.* repress) the product formation as well the AOX activity. Continuously added methanol served mainly to induce and maintain product formation; simultaneously added glucose did not accumulate in the culture broth and was immediately utilised as both growth- and energy-substrate.

Our results with glucose and methanol mixtures suggest the following generic principle of repressor elimination in methylotrophic yeasts: provided neither carbon substrates nor metabolites accumulate in the culture broth, simultaneous utilisation of substrate mixtures in carbon-limited cultures prevents 'repressive' substrates from lowering product formation under tight AOX-control. Thus, this principle tallies with data from chemostat cultures published in the 1980s on wild-type *Hansenula polymorpha* with glucose and methanol mixtures (*i.e. Pichia augusta*, Egli *et al*., 1986) as well with later studies on *Pichia pastoris* with mixtures of glycerol and methanol (Brierley *et al*., 1990; Jungo *et al*., 2007; Sola *et al*., 2007).

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Sola, A., Jouhten, P., Maaheimo, H., Sanchez-Ferrando, F., Szyperski, T., and Ferrer, P. (2007) Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. Microbiol. 153, 281-290.

6. Expression of four different laccases from *Trametes versicolor* in *Pichia pastoris*

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Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2.) are very attractive enzymes for biocatalysis as they work with molecular oxygen at ambient temperatures, produce water as a by-product and do not require the costly co-factors NADH or NADPH as many other oxidoreductases. They belong to the group of multicopper oxidoreductases and oxidize electron-rich compounds such as phenols, thiols and N-hydroxyl groups by radical mechanism through the concomitant reduction of molecular oxygen to water (Thurston *et al.,* 1994). Laccases from the white rot fungus *Trametes versicolor* belong to the well studied laccases. A phylogenetic analysis of the laccases from *T. versicolor*, whose sequences have been reported so far, revealed four isoenzyme groups α, β, γ and δ (Necochea *et al.,* 2005). We cloned all four laccase genes (*lccα, lccβ, lccγ and lccδ*) either with their native signal peptides or with the α-mating factor signal peptide of *S. cerevisiae* into *S. cerevisiae* expression vector *pYES2*. However, expression in *S. cerevisiae* InvSc1 cells was quite low. Expression of laccases in protease-deficient hosts sometimes improved expression level of these enzymes (Jonsson *et al.,* 1997). Using the protease-deficient strain YWO 0247 of *S. cerevisiae* expression levels of Lccα and Lccγ were up to 5 fold higher than with InvSc1. Nevertheless, volumetric activities reached just 1 – 2 U/l. Therefore, laccase genes were cloned into *P. pastoris* expression vector *pPICZA* under control of the *AOX1* promoter and inserted into *P. pastoris* X-33 cells. Expression was much higher compared to *S. cerevisiae*. Recombinant expression and secretion of laccases in *P. pastoris* were highly affected by cultivation temperature and signal peptide used. Volumetric activities up to 105 U/l of Lccβ towards ABTS were reached in shake flasks and up to 3400 U/l of Lccβ in fed-batch fermentations. However, expression of the laccases in protease-deficient strain SMD1168H of *P. pastoris* resulted in lower volumetric activities compared to the wildtype strain. Biochemical characterization of the four laccases clearly showed that Lccα and Lccβ possess similar biochemical properties, obviously different from Lccγ and Lccδ. Regarding their enzymatic properties these four laccases can also be strictly distinguished. Lccδ showed highest activity in dimerization of sinapic acid and Lccα lowest activity in this reaction. Highest oxidizing activity towards polycyclic aromatic hydrocarbons (PAHs) was observed with Lccβ, whereas Lccδ showed very low activity.

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7. Use of site-specific recombination to create a *Pichia pastoris* Mut^{s} strain

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S. cerevisiae site-specific recombination mechanism employs FLP recombinase and two asymmetric FLP recombination target sequences (*FRTs*) derived from *S. cerevisiae* 2 µm circle (Broach *et al*., 1981; Som *et al*., 1988). Orienting two *FRTs* as direct repeats results in precise *FLP* protein mediated deletion of all DNA between the *FRTs* (Vetter *et al*., 1983). The system has previously been reported to function in *P. pastoris* by first inserting *FRTs* with a selection marker to the genome and transforming *FLP* on a separate plasmid (Cregg *et al*., 1989). A simplified version of the method to introduce all parts needed to the genome in a single transformation step has been reported to function in *C. albicans* (Reuss *et al*., 2004). This report describes the assembly and integration of a single cassette to specifically knock out the coding sequence of *Pichia pastoris* alcohol oxidase (*AOX1*) gene from wild type *Pichia pastoris* strain without leaving any cassette components except one *FRT* (34bp) behind in the genome.

Broach, J.R. (1981) The yeast plasmid 2 µm circle. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.), The molecular biology of the yeast *Saccharomyces:* Life cycle and inheritance. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 455-470.

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8. A kit for fast and efficient expression and directed evolution of proteins in *Pichia pastoris*

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Most often, bacteria, especially *E. coli*, are used for heterologous protein expression and subsequent laboratory evolution, because of the existing well-known and simple toolbox for molecular genetic manipulation, high transformation efficiency and rapid growth rates. For eukaryotic proteins, however, the use of prokaryotic expression systems is often limited due to misfolding and the lack of enzyme machineries which perform typical eukaryotic posttranslational modifications. *Pichia pastoris*, an efficient eukaryotic alternative for heterologous protein production (Cereghino *et al*., 2000), can compensate for such disadvantages. We developed a new kit for fast and efficient construction of expression libraries in *P. pastoris* providing uniform expression of thousands of transformants and circumventing time-consuming ligation and cloning steps in *E. coli* which reduce diversity and library efficiency tremendously. To test the strategy, linear expression cassettes were generated linking together three DNA fragments: a 5' arm containing the promoter and a secretion signal sequence, a mutated target gene and a 3' arm with the selection marker cassette. Both flanking arms were made by proofreading PCR. The full linear expression cassette was assembled by overlap extension PCR. Then, without any prior ligation step, *P. pastoris* was directly transformed with the linear PCR-based integration cassettes (Liu *et al*., 2008). In addition, we made this new PCR-based strategy more generally applicable. It is made for constitutive and inducible expression and enables the linkage of any *Pichia pastoris* promoter with any desired native, synthetic or mutated gene for intra- or extracellular expression and a marker of choice.

Cereghino, J.L. and Cregg, J.M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45-66.

Liu Z., Pscheidt, B., Avi, M., Gaisberger, R., Hartner, F.S., Schuster, C., Skranc, W., Gruber, K. and Glieder, A. (2008) Laboratory evolved biocatalysts for stereoselective syntheses of substituted benzaldehyde cyanohydrins. Chem. Bio. Chem. 9, 58-61.

9. c-LEcta's *Pichia* expression platform for the screening and production of enzymes

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c-LEcta has build up on own expression system based on *Pichia pastoris*. Efficient production strains and the necessary vectors are available in the company. The system allows the production of highly efficient multi-copy integrants for the industrial production of enzymes with very high yields. The technology covers several promoters with different strength all of which are independent from methanol induction allowing finding the optimal expression system for a particular enzyme. The optimization of a production strain is facilitated by efficient strategies for multi-copy integration in combination with c-LEcta's screening expertise. In addition c-LEcta build-up a system which allows high transformation efficiency and uniform expression level, which is crucial for a screening system. C-LEcta uses this eucaryotic screening system for enzyme optimisation and identification of new enzymes in natural diversity. Once an optimal enzyme is identified it can be readily transformed into an efficient industrial production strain.

10. Novel promoters for protein production in *Pichia pastoris*

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The yeast *Pichia pastoris* is commonly used as host system for heterologous protein production (Cereghino *et al*., 2000). However, compared to other yeast species *e.g. Saccharomyces* the number of useable promoter sequences is rather small and also limited to promoters having a very strong activity. For some applications, *e.g.* the co-expression of secretion helpers, it might be of interest to use promoters with lower transcriptional levels. It is therefore useful to have a selection of different promoter sequences suitable for recombinant expression of a heterologous or homologous gene, varying from strong promoter activity to weak or reduced promoter activity as compared to the *GAP* promoter. In order to identify novel promoter sequences for use in *P. pastoris*, the data derived from the heterologous microarray hybridisation of *P. pastoris* cDNA to *S. cerevisiae* cDNA microarrays (Sauer *et al*., 2004) were evaluated in a specific manner. From these data the 23 genes with the highest expression level were considered for further analysis, indicating that they possess effective and strong promoter sequences. The potential promoter regions (up to 1000 bp of the 5'-region) of these genes were amplified from *P. pastoris* by PCR and cloned into a *P. pastoris* expression vector, which carries either an enhanced green fluorescent protein (eGFP), human serum albumin (HSA) or beta-galactosidase (lacZ) as a reporter gene. To test the properties of the different promoters, i.e. the promoter activity, the 25 vectors (including the *AOX* and *GAP* promoter of *P. pastoris* as reference) were subsequently transformed into a *P. pastoris* strain. Promoter activities were determined indirectly by measurement of the amount of gene product expressed from the promoter. In the present study, clones expressing two intracellular re-porters (eGFP and beta-galactosidase) and one secreted heterologous protein (HSA) were cultivated under different culturing conditions and the amount of recombinant reporter protein was quantified using flow cytometer analysis, enzyme activity assay, and ELISA, respectively. A comparative analysis of the novel 23 promoter sequences and the well established *P. pastoris* promoters (*pGAP* and *pAOX*) was performed. Under chosen conditions the promoter activities ranged from 0 up to 700% compared to *pGAP*/*pAOX* promoter activity, allowing the use of tailored promoter sequences for different needs or applications. This allows to regulate the expression level of a protein of interest by selection of a suitable promoter sequence according to the experimental situation.

Cereghino J.L. and Cregg J.M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 4.

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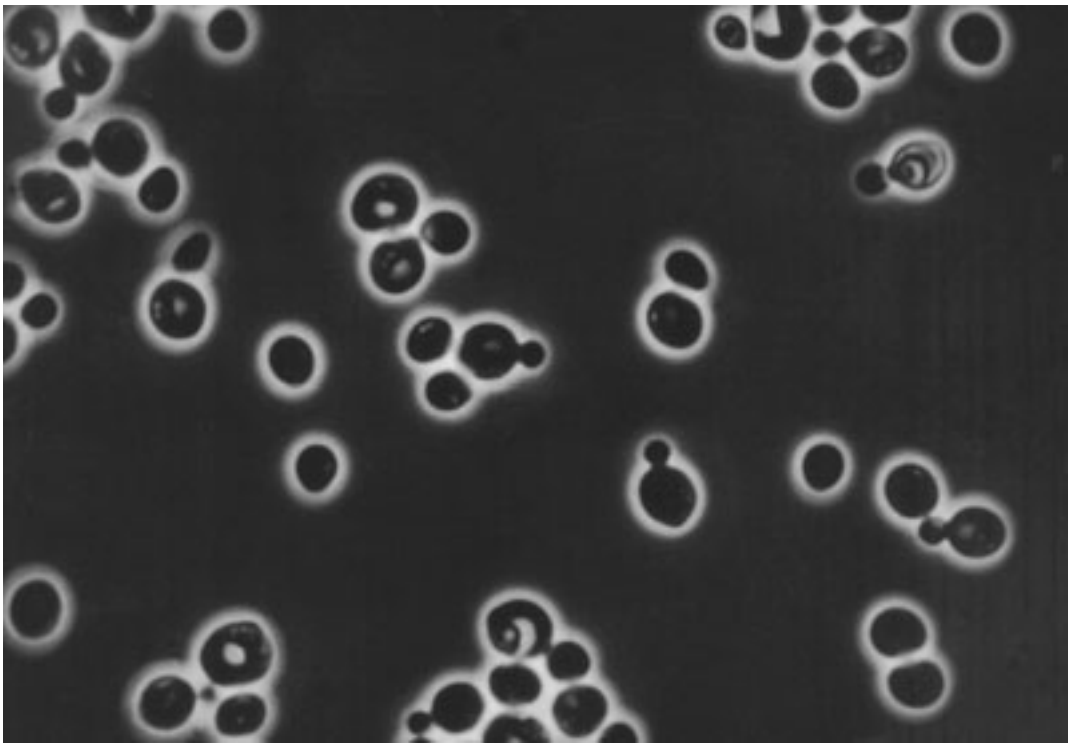

We started with the *Pichia pastoris* Yeast Protein Expression System in 1993.

Today, our Gene eXpression Program provides an array of the world's most widely used gene expression systems, tools, genes and antibodies through one convenient and affordable licensing program.

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11. Vitality of *Pichia pastoris* yeast during heterologous protein production phase

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Being yeast physiology a key parameter for protein overexpression, vitality of cells can highly influence their production ability, an expression rate and a quality of excreted protein. Therefore, there is an interest to measure and control this parameter in an effort to maximize number of actively producing cells and to prevent desired protein from degradation caused by the presence of proteases released from dead cells.

In this study fluorescent viability staining in connection with flow cytometry was used to find out relations between cultivation conditions, production rate and cell viability of Mut^+ *Pichia pastoris* strain excreting (upon methanol induction) recombinant porcine trypsinogen. Comparative screening of different fluorescent probes was performed to determine their ability to resolve viable and dead *Pichia pastoris* cells on the basis of detection of following cell characteristic: membrane integrity using ethidium bromide (EB), propidium iodide (PI) and Sytox green; transmembrane potential employing Rhodamine 123, 3,3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$) and bis-oxonol (BOX); intracellular esterase activity applying fluorogenic enzyme substrates fluorescein diacetate (FDA) and carboxyfluorescein diacetate (CFDA). Since the uptake rate of the perfluorochromes FDA and CFDA was very low and $DiOC_2(3)$, Rhodamine 123, EB and Sytox green were suitable viability marker only under specific conditions. Finally two fluorescent probes, propidium iodide and bis-oxonol were chosen as the most appropriate and applied to distinguish healthy yeasts having intact and polarised cytoplasmatic membrane, respectively.

Chemostat cultures were grown at different dilution rates on mixed glucose and methanol medium as a feed. It was expected that a high cell density, protein overexpression, release of the active product form (trypsin) and residual methanol in culture broth could act as cellular stress factors and induce loss of cell vitality. However, only the residual methanol concentration was proved to have significant influence on *Pichia pastoris* viability. Presence of stained cells (PI ~2% and BOX ~3%) remained constantly low during the period with the maximum specific heterologous protein productivity and varied slightly showing no remarkable dependence on the concentration of trypsinogen achieved.

12. Recombinant protein production in *Pichia pastoris* using byproduct of biodiesel production

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The rapidly growing biodiesel production industry is in urgent need for the development of new routes to convert their byproduct crude glycerol, which is now considered as a waste, into higher value products. Since biodiesel is produced from vegetable oils by transesterification with methanol, the glycerol phase of transesterification reaction (crude glycerol) contains residual methanol, as well as sodium hydroxide, carry-over fat/oil, some esters, low amounts of sulfur compounds, proteins, and minerals in addition to glycerol; thus, the costly purification step is inevitable prior to the use of crude glycerol in food, pharmaceutical, and cosmetic industries. Using crude glycerol in fermentation processes as the carbon source is another option; however, methanol has to be removed as it is highly toxic for most of the microorganisms. On the other hand, a microorganism that can tolerate methanol can be used to avoid the methanol removal step. The microorganism *Pichia pastoris*, a methylotrophic yeast (yeast that can grow on methanol), can tolerate high methanol concentrations and can utilize glycerol effectively. Moreover, *P. pastoris* is commonly used as a host in industry for the production of various recombinant proteins, because it produces foreign proteins at high levels.

In this context, the aim of this study was to investigate the efficiency of using crude glycerol, the main byproduct of biodiesel industry, directly in the production media of *P. pastoris* fermentation processes. The system designed for production of the therapeutically important glycoprotein, recombinant erythropoietin, by *P. pastoris* was used (Çelik *et al*., 2007). Different biodiesel synthesis conditions were examined, and although some crude glycerol samples were found to be inefficient fermentation medium components due to their tendency to form potassium and sodium salts of free fatty acids, canola oil-derived glycerol byproduct of biodiesel served as a beneficial carbon source to be used in the production of additional value-added products, without any purification. At a 1:6 molar ratio of canola oil:methanol and C_{NaOH}=1% (w/v), the highest product yield on substrate ($Y_{rEPO/S}$ = 1.48 mg/g), and cell yield on substrate ($Y_{X/S}$ = 0.57 g/g) were obtained, having values approximately 1.3-fold higher than those attained with pure glycerol, in shake flask cultures (Çelik *et al*., 2008). This is likely due to the presence of fatty acids, vitamins A, E, and K and trace elements in the vegetable oil diffusing to the glycerol phase during the biodiesel formation reactions, and thus enriching the glycerol-based production medium.

This study shows the convergence of two rapidly growing industries, the biodiesel and the fermentation process industries, where *P. pastoris* plays a crucial role being a methylotrophic microorganism.

Çelik, E., Çalık, P., Halloran, M. and Oliver, S.G. (2007) Production of recombinant human erythropoietin from *Pichia pastoris* and its structural analysis. J. Appl. Microbiol. 103, 2084-2094.

Çelik, E., Özbay, N., Oktar, N. and Çalık, P. (2008; in press). Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris.* Ind. Eng. Chem. Res. *doi: 10.1021/ie071613o*

13. Design of a production process for the plant enzyme XET in *Pichia pastoris*

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The aim of this work was to design a production process for the enzyme xyloglucan endotransglycosylase (XET, EC 2.4.1.207). The natural transglycosylating activity of this plant enzyme has been employed in the development of a XET-Technology (Brumer *et al*., 2004). In this chemo-enzymatic approach, a variety of cellulose surfaces and materials can be biomimetically modified with a wide range of different chemical groups (Zhou *et al*., 2005 and 2006). The XET-Technology holds great potential for industrial applications. However, it requires that the enzyme can be produced in much larger scale. A heterologous production system was previously established by the lab in the methylotrophic yeast *Pichia pastoris* (Kallas *et al*., 2005). A methanol-limited fedbatch was also developed but the yield of active enzyme in this process was low due to proteolysis problems and low productivity (Bollok *et al*., 2005). In this work, we investigated two alternative fedbatch processes for the production of XET: a temperature-limited fedbatch (TLFB) and an oxygen-limited high-pressure fedbatch (OLHPFB). By combining these techniques; we could increase the production of recombinant XET by 24% per reactor. For the initial product recovery two different downstream processes were investigated: crossflow filtration (CFF) and expanded bed adsorption (EBA). These techniques were compared on parameters such as yield of active enzyme, product quality, number of handling steps and total required process time.

Bollok, M., Henriksson, H., Kallas, Å.M., Jahic, M., Teeri, T.T. and Enfors, S.O. (2005) Production of poplar xyloglucan endo-transglycosylase using the methylotrophic yeast *Pichia pastoris*, Appl. Biochem. Biotechnol. 126, 61-77

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14. Determination of physiological changes during fedbatch processes with *P. pastoris* expressing recombinant proteins

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The key to maximising product formation in biotechnological processes is the ability to rapidly monitor and interpret the physiological state of microorganisms. In contrast to conventional process monitoring (routinely used in industrial manufacturing and/or research to determine uniformly distributed values within an entire population), flow cytometric methods (FCM) enable measurements of physiological state to be performed rapidly and at both individual cell and subpopulation levels. The potential use of FCM in maximising process performance has been explored in high-cell-density (HCD) fedbatch cultures of *Pichia pastoris* Mut$^+$ extracellularly secreting different recombinant products (e.g. porcine trypsinogen, horseradish peroxidase, glycoproteins such as mGM-CSF) and compared to control experiments with non-producing wild type X33 strain. Subtle changes in cell physiology were indicated by the gradually increasing and/or decreasing the proportion of cells stained by fluorescent dyes (e.g. propidium iodide, ethidium bromide, carboxyfluorescein diacetate, bis-oxonol).

The most pronounced change was detected at a late stage in the production process of porcine trypsinogen at pH 4 and 30 °C using mineral medium with a constant addition of a mixture of glucose and methanol (i.e. at 30 h after induction at cell densities > 60 g/l CDW over 80 % of the 'aged' cells had both compromised membranes and defective transport systems, and in less than 20 % of the cells enzyme activity was detected; subsequently, an accumulation of methanol in culture broth up to 80 g/l was observed). The changes determined in the permeability, transport and/or polarity of cell membranes and/or activity of intracellular enzymes, as well as a general reduction in vitality, were caused by the combined effect of (i) exposing cells to an extracellular concentration of methanol or proteases, (ii) the composition of culture media and their pH, and (iii) the particular heterologous protein expression. In addition, several control experiments revealed that cell vitality improves when any one of these factors is excluded. Furthermore, significant differences in cell sensitivity to methanol were observed when cells were taken from different phases of a fedbatch process and incubated for 5 h with methanol concentrations up to 200 g/l: Cells already induced for protein expression changed to a higher percentage of the total population than those assayed before induction by methanol and cells grown at pH 4 showed more pronounced methanol-sensitivity than those cultured at pH 6.

As our results tally with interpretations of physiological changes described by Jungo *et al.* (2007) and Gasser *et al.* (2008), the flow cytometry method applied shows promise with respect to the further development of early-warning systems to prevent sudden decreases in productivity.

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15. Screening and production of *Candida antarctica* lipase B in *Pichia pastoris*; inducible versus constitutive expression

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Protein expression in the yeast *Pichia pastoris* was investigated using, as a model system, *Candida antarctica* lipase B fused to a cellulose binding module. The well known inducible *AOX1* promoter was compared to the constitutive *GAP* promoter. Expression of lipase was evaluated in cultures growing on different carbon sources: methanol using the *AOX1* promoter, glucose and glycerol using the *GAP* promoter. In addition, cell viability and proteolysis were measured. In shake flask cultivations the *GAP*/glycerol system proved superior to the other tested systems, yielding 37 mg lipase per litre in 72 hours. This was scaled down to a 96-deep-well plate format using glycerol as a carbon source. This system proved to be very efficient, yielding 37 mg lipase per litre in 28 hours. The *GAP* promoter was found suitable for protein production in both deep-well and shake flask making it a valuable tool for screening and small scale production. For production purposes the productivity was evaluated in bioreactor cultivations. Up-scaling to bioreactor resulted in 1900 mg/L using *AOX1*/methanol, 1500 mg/L using *GAP*/glycerol and 600 mg/L using *GAP*/glucose. Significant differences in the total volume and cell density between the three systems were found and must be accounted for in the evaluation of the systems.

16. Production of recombinant protein and physiology of *Pichia pastoris* Mut$^+$ strain grown in continuous culture

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Continuous culture (chemostat) was applied as a tool for a study of relationship between dilution rate, overexpression of excreted recombinant trypsinogen and physiology of *Pichia pastoris* Mut$^+$ strain grown on mixed substrate containing two carbon sources - glucose and methanol. Processes employing mixed substrate for heterologous protein production are mentioned by various authors (Ihnan *et al*., 2001; Xie *et al*., 2005; Cos *et al*., 2006) to be superior to processes using methanol as sole carbon source especially for lower heat evolution rate, lower oxygen consumption rate and higher cell densities. The maximum specific growth rates for *Pichia* Mut$^+$ strain grown on mixed substrate $(0.269 \pm 0.003)\ h^{-1}$ or on methanol $(0.115 \pm 0.001)\ h^{-1}$ determined in wash-out experiments in this study can prove the last mentioned information. Although glucose is considered to be repressive substrate for *AOX* promoter (Sauer *et al*., 2004), recombinant trypsinogen was expressed when glucose was used in mixture with methanol in substrate limited culture. The specific methanol uptake rate was increasing along with increasing dilution rate reaching its maximum (0.09 ± 0.01) g/(gCDW h) at dilution rate $0.15\ h^{-1}$; both co-substrates were fully utilized under this condition. At dilution rates exceeding $0.15\ h^{-1}$ the specific methanol uptake rate was decreasing and a residual methanol was detected in the culture broth. On the other hand, the specific glucose uptake rate was increasing within the interval of measured dilution rates reaching maximum (0.24 ± 0.01) g/(gCDW h) at $0.2\ h^{-1}$. This is in agreement with results published by Egli *et al*. (2004) - in case of continuous cultures performed at higher dilution rates the cell culture prefers the higher carbon source and unutilised methanol is accumulated. Also the specific productivity of recombinant protein in chemostat culture grown on mixed substrate was found to be influenced by dilution rate. Both maximum concentration of porcine trypsinogen (145 mg/l) and maximum specific productivity (1.17 mg/(gCDW h)) were achieved at dilution rate $0.13\ h^{-1}$. The physiological response of host strain to overproduction of recombinant protein and culture condition was determined using flow cytometry together with fluorescent staining of selected physiological markers – integrity of cytoplasmatic membrane (stained with cell impermeable dye propidium iodide) and it's polarization (marked with anionic probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol). The physiology of host culture was found to be influenced rather by presence of residual concentration of methanol then by high specific productivity of recombinant protein. When maximum specific productivity of trypsinogen was achieved, 2% of cells with compromised membranes and 3% of depolarized cells were measured compared to 6% and 9% of cells stained with propidium iodide and bis-(1,3-dibutylbarburic acid) trimethine oxonol when 6 g/l of residual methanol was detected in the culture broth.

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17. High throughput screening for factors enhancing protein production and secretion with *Pichia pastoris*

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Efficient production of heterologous proteins with yeasts and other eukaryotic hosts is often hampered by inefficient secretion of the product. Limitation of protein secretion has been attributed to a low folding rate, and a rational solution is the overexpression of proteins supporting folding, like protein disulfide isomerase (PDI), or the unfolded protein response transcription factor *HAC1*. However, all these approaches are limited to the existing knowledge base. Novel processes might be identified and targeted to improve secretion (including genes not directly involved in folding) through different approaches. Due to the limited physiological knowledge about *Pichia pastoris* there is a demand for high throughput methods for identification of bottlenecks in the production of heterologous proteins. In this regard, high throughput flow cytometry and cell sorting are valuable tools to isolate overproducing clones. One approach is to screen overexpression libraries for improved secretion of heterologous protein, which is anchored to the cell surface via agglutinin and detected by immunofluorescent staining. The present work describes the use of fluorescence activated cell sorting (FACS) as a high throughput system to isolate novel helper factors out of cDNA coexpression libraries to increase the production of Fab antibody fragments in *P. pastoris*. A cDNA pool, obtained from *Pichia* strains grown under different metabolic und environmental conditions, is coexpressed in a strain carrying a human antibody Fab fragment fused to the *SAG1* membrane anchor domain of *Saccharomyces cerevisiae*. FACS is used to enrich cells displaying an increased immunofluorescent cell surface signal. In a first very stringent sorting experiment three different genes could be identified, which have never been described before to have an effect on protein secretion.

18. Citric acid production from sucrose by *Yarrowia lipolytica* recombinant strains

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The *hemiascomycetous* yeasts *Yarrowia lipolytica* become one of the most attractive organisms in the field of applied biotechnology and it has been used as an eukaryotic model organism for the study of protein secretion, carbon metabolism, peroxisome biogenesis and regulation of gene expression. This species growth on large spectrum of hydrophobic substrates, secrete various enzymes (proteases, lipases or RNase), but they do not utilize sucrose and any of disaccharides. A wild type, strain *Y. lipolytica* A-101, isolated from soil in Poland have been intensively studied for citric acid biosynthesis on different substrates. Recently, we have obtained 337 transformants as a result of transformation of *SUC- URA+ Y. lipolytica* A-101 wild type strain by yeasts expression/deletion cassette with *SUC2* gene under *XPR2* promoter. Stable homologous integration of used sequence was observed for 4 of obtained modified strains (phenotype *SUC+URA-*). Heterologous integration sites (*SUC+URA+*) are under investigation. The aim of this study was the comparison of citric acid production by *SUC+URA-* and *SUC+URA+ Y. lipolytica* A-101 tranformants. In Bioscreen study on MMT medium (sucrose 1%, peptone 0.1% and uracil, when necessary) the best growth of *Yarrowia lipolytica SUC+URA+* transformants, resulting in heterologous integration of expression/deletion cassette was observed (OD_{550} 1.5-1.7). The growth of *Y. lipolytica SUC+URA-* transformants, resulting in homologous integration of expression/deletion cassette was smaller (OD_{550} 0.2-0.4). During flask Batch Culture (BC) of selected transformants on 10% sucrose medium, a moderate citrate secretion (5-10 g/L) was observed (HPLC analysis). For tree of transformants (*Y. lipolytica* B56-5, *Y. lipolytica* B57-4, *Y. lipolytica* A18) a 2 L bioreactor (Biostat) citrate biosynthesis from sucrose was investigated. During the culture organic acids, fructose, glucose and sucrose were measured on HPLC and invertase activity was determined in broth and cells. Differences in growth rate, sucrose and monosaccharides consumption rate and in citrate productivity were compared to invertase activity expression.

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Thanks to Switzerland's long-standing industrial tradition in the pharmaceutical, chemical and Life Science fields, it is the home of roughly 240 Biotech companies that value a business environment supporting rapid, solid growth in Europe. In many instances, these firms are cutting research leaders in their respective markets. Together, they employ a full-time workforce of over 14'000; they also invest well over 1 billion USD annually for research and development.

The Swiss biotech industry is among the largest and most diversified in Europe. The central position of Switzerland provides a platform to access the European market with more than 450 million consumers of products and services. With Europe's highest per capita income, Switzerland is not only attractive and innovative, but it represents a demanding market with one of the best health care systems in the world, encompassing an extraordinarily high density of hospitals and clinics. Good contractual and regulatory relations with the EU and the central geographic position provide Switzerland-based companies with virtually unrestricted access to the fast-growing Biotech market world-wide.

Furthermore, the biotech industry has developed over the past years into an internationally recognised sectoral focal point and is tracked by an active public and private investor base. Today, the representatives of the industry listed on the Swiss Stock Exchange (SWX) collectively represent the largest peer group of its kind in Europe in terms of market capitalisation. Differentiated sector-specific indicators such as the SXI LIFE SCIENCES® and SXI Bio+Medtech® indices, as well as the extraordinarily broad analyst coverage of this particular sector, ensure transparency and further enhance the outstanding degree of visibility. For these reasons, companies seeking additional funding will find a highly knowledgeable and experienced investor base in Switzerland and on the SWX.

Switzerland is 'one nation, one biotech cluster'. The national cluster is promoted internationally by the Swiss Life Science Marketing Alliance – a joint initiative of the regional clusters BaselArea, bio-alps, Biopolo Ticino, Greater Zürich Area and the Swiss Stock Exchange, initiated by the Swiss Biotech Association, the national industry association.

Extensive details on the Swiss biotech cluster can be found on the web portal www.swissbiotech.org, in the publicly open company database at www.swisslifesciences.ch, and in the Swiss Biotech Report, freely available at http://www.swissbiotech.org.

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19. Recombinant human growth hormone production in *Pichia pastoris*

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Human growth hormone (hGH) is anionic, non-glycosylated protein known as somatotropin, having a molar mass of 22 kDa and 191 amino acid residues. It has been used to treat hypopituitary dwarfism, injuries, bone fractures, bleeding ulcers, and burns. Recently it appears to be of considerable benefit to girls with Turner's syndrome, children with chronic renal failure and adults with growth hormone deficiency or human immunodeficiency virus (HIV) syndrome. In this study, we have constructed the elements for the production and affinity purification of recombinant proteins expressed in a *Pichia pastoris* host (Çelik *et al*., 2007). The *hGH* gene was joined with a polyhistidine tag; the presence of the tag enabling use of metal affinity chromatography to obtain the recombinant protein in high purity. In addition, a target site for the Factor Xa protease was fused in such a way that cleavage produces a mature form of rhGH having only the native sequence with no extraneous tags. The plasmid containing the constructed elements, *pPICZαA::hGH*, under the control of the alcohol oxidase promoter, was integrated to the *AOX1* locus of *P. pastoris* (Çalık *et al*., 2008). After digestion with Factor Xa protease, rhGH with entirely the native sequence was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to show the effectiveness of the expression. Analyses of the affinity-purified rhGH product by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) showed a spectral peak at *m/z* 23699. Purified product digested with Factor Xa protease had a molecular weight of 22132 kDa. The molecular weight difference before and after Factor Xa protease digestion expectedly corresponds to the 12 amino acids in the rhGH amino terminus, which includes the *Eco*RI digestion site (Glu-Phe), the 6xHis tag for affinity-purification, and the Factor Xa protease recognition sequence (Ile-Glu-Gly-Arg), a result that also indicates that the signal peptide was properly processed by *P. pastoris* (Çalık *et al*., 2008). N-terminal sequence analysis of the Factor Xa protease trimmed recombinant product confirmed the mature hGH sequence. Thus, the system designed, functioned with its intended purpose effectively in expression, cleavage and purification of the recombinant product.

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20. Alpha-L-Fucosidase and beta-D-fucosidase activities of *Paenibacillus thiaminolyticus*

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Glycosidases are enzymes, which are involved in metabolism of oligo- and polysaccharides, glycoproteins and glycolipids and thus their role for the living organisms is unique and irreplaceable. Saccharidic structures are involved in many important biological processes, for example cell-cell interaction, adhesion, morphogenesis and differenciation, but also inflammation or metastatic spread of tumor cells. This fact is nowadays the start point for the progressive evolving part of biochemical and pharmaceutical research, which is interested in the influence of glycosylation on the properties of different biological active molecules. One possibility how to synthetise new glycosylated molecules for the biochemical testing, is the usage of glycosidases with the ability to catalyze transglycosylation reaction. As the monosaccharide alpha-L-fucose is a part of saccharidic chains of different receptors on the cell surface and because it is also present in glycoconjugates, which play important role as antigenic determinants and are therefore necessary for correct function of immune system, the enzymes, which are able to cleave the fucosyle residue from the saccharidic chains, are now under very intensive investigation. The information about the transglycosylation abilities is important for the research, because these enzymes could be used for preparation of different biological active molecules as for example drugs or inhibitors. In this work, focused on alpha-L-fucosidase and beta-D-fucosidase abilities of bacteria, several bacterial strains were screened for alpha-L-fucosidase activity. The only strain, which was able to cleave the chromogenic substrate p-nitrophenyl-alpha-L-fucopyranoside, was *Paenibacillus thiaminolyticus*. In this bacteria alpha-L-fucosidase activity as well as the beta-D-fucosidase activity was detected and this bacteria was thus chosen for the next work. The enzyme alpha-L-fucosidase was characterized and purified using different chromatographic and electrophoretic techniques. During the purification process two isoenzymes were detected. N-terminal amino-acid sequence was determined for one of them, but the abilities to catalyze transglycosylation reactions using nonsaccharidic acceptor with antiviral activity were tested for both isoenzymes. Simultaneously, it was determined that the beta-D-fucosidase activity could be the second activity of the beta-D-galactosidase of *Paenibacillus thiaminolyticus*. The gene for this enzyme was found and sequenced and the presumption was confirmed. Recombinant enzyme was produced using *E. coli* BL21 cells, purified using the affinity chromatography and basic characteristics were measured. In the future the ability to catalyze transglycosylation reaction using different natural and unnatural donor molecules and saccharidic or non-saccharidic acceptors will be tested for the beta-D-galactosidase with beta-D-fucosidase activity.

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21. Engineering of the *Yarrowia lipolytica* protein expression system

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High performance expression systems are required to produce all biopharmaceuticals that are currently under development to fulfil the future need for recombinant proteins. Yeasts combine the ease of genetic manipulation and fermentation of a microbial organism with the capability to secrete and to modify proteins according to a universal eukaryotic design. However, a downside of using yeasts as a production host for the therapeutic glycoproteins is the nature of their glycans. N-glycosylation starts in the endoplasmic reticulum with the synthesis of a dolichol linked precursor structure. In the Golgi apparatus of yeasts, the $Man_8GlcNAc_2$ N-glycans are further extended by the addition of mannose and phospho-mannose residues. The presence of heterogenous high-mannose structures can be detrimental for therapeutic protein efficacy and/or downstream processing efficiency. In contrast, glycans in higher eukaryotes are first trimmed to $Man_5GlcNAc_2$ and then further modified to complex type glycans.

Yarrowia lipolytica is a GRAS-status dimorphic yeast that efficiently secretes various heterologous proteins and can be cultivated to high cell densities in large-scale fermenters. It is thus an attractive host for protein production. We engineered the secretory pathway of *Yarrowia lipolytica* to develop strains that produce homogenously glycosylated recombinant proteins. Here, we describe how we interfered with the endogenous core N-glycan biosynthesis in *Yarrowia lipolytica*, to obtain a homogenous $Man_3GlcNAc_2$ structure on its glycoproteins. This is the ideal starting point to build up human-like sugars.

This work has been performed as a collaborative project with Oxyrane UK Ltd.

22. Characterization of the *HAC1* gene from *P. pastoris* and evaluation of the effect of its overexpression on the secretion of heterologous proteins

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Accumulation of unfolded and misfolded proteins in the endoplasmic reticulum triggers the activation of the unfolded protein response (UPR). In *Saccharomyces cerevisiae* UPR is activated by a non-conventional splicing event of the *HAC1* mRNA. The non spliced *HAC1* mRNA is translationaly attenuated due to secondary RNA structures and does not code for a functional Hac1p protein whereas the spliced *HAC1* mRNA encodes for an active transcription factor. The latter binds to UPR-responsive elements in the promoter of UPR target genes which encode for chaperones, foldases, proteins involved in lipid and inositol metabolism, etc. This protective mechanism enables cells to recover from this stressful event, whereas absence of UPR can eventually result into cell death. We identified the *HAC1* homologue of *Pichia pastoris* and showed that the *P. pastoris HAC1* mRNA undergoes an analogous splicing reaction. The intron-free *HAC1* cDNA was cloned under control of a strong inducible promoter. After induction of the expression of the spliced *HAC1*, UPR was quantified by evaluation of the expression of the chaperone KAR2, the best characterized UPR-target gene. By means of Q PCR, we could demonstrate a 35 fold upregulation of the foldase KAR2. Constitutive overexpression of the *HAC1* gene of *S. cerevisiae* can lead to an increased production of native and heterologous secreted proteins. In order to check if this also holds true for the Hac1p transcription factor of *P. pastoris* a dual approach was followed. First, Hac1p was cloned in a mouse IL-10 expressing *P. pastoris* strain. We showed that indeed, overexpression of *P. pastoris* Hac1p leads to a ~2 fold increased expression of the mouse IL-10 protein. Second, we show that overexpression of Hac1p can lead to increased surface expression of a heterologous protein, indicating again the beneficial effect of *P. pastoris* Hac1p overexpression on protein secretion. In this validated approach, FACS analysis of surface expression levels allows for easier quantification of recombinant protein expression as compared to soluble expression.

23. Production of humanized therapeutic proteins in the yeast *Pichia pastoris*

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Protein-based therapeutics are emerging as the largest class of new entities being developed by the drug industry. About 70% of all approved therapeutic proteins on the market are glycoproteins. The production of recombinant proteins that are N-glycosylated has in most cases required mammalian expression hosts that have the ability to synthesize human-like N-glycans. However, purified proteins from a mammalian cell culture process are essentially a mixture of individual drugs, some of which are more active than others and some of which have no activity at all. Each of these glycoforms has its own pharmocokinetic, pharmocodynamic and efficacy profile. The methylotrophic yeast *Pichia pastoris* is an excellent alternative for the production of therapeutic glycoproteins: the system is cheap, the yields are high and there is no risk for viral or prion contamination. However, the N-glycosylation is non-human, heterogeneous and may cause immmunogenic reactions. We are currently tackling these handicaps by engineering the *P. pastoris* N-glycosylation pathway to allow the synthesis of human-like oligosaccharides. The general strategy consists in knocking out endogenous glycosyltransferases (responsible for the synthesis of yeast specific N-glycans) and introducing heterologous glycosidases and glycosyltransferases (responsible for the build-up of mammalian-type N-glycans). In this way we have created a library of yeast strains that have been engineered to modify all proteins with one specific N-glycan. Recently, we have started producing several therapeutically interesting glycoproteins in these strains and the characteristics of the individual glycoforms will be tested both *in vitro* and *in vivo*.

24. Cell wall-anchored mannoprotein variants engineered to enhance metallosorption by *Saccharomyces cerevisiae*

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Biosorption of heavy metals is a potent tool with significance for recuperation of metals in the industry and for cleanup of contaminated waste water to meet regulatory agency limits. We employed the carboxyl-terminus of the sexual adhesion glycoprotein alpha-agglutinin (AGalpha1Cp) as an anchoring domain to achieve surface display of the short metal binding peptides on cell walls of the yeast biosorbent. CP2 peptide $S(GCGCPCGC)_2G$ and HP3 peptide $S(GHHPH)_3G$, shown to promote metallosorption by *E. coli* cell wall (Sousa *et al.*, 1998), and/or the metal fixation motif of P1-ATPases of sequence MDCPTEEALIR (NP peptide) were genetically fused to AGalpha1Cp, expressed fusions were found covalently attached to cell wall glucan of *S. cerevisiae* at 4.5 × 106 copies per cell and glycosylated (Vinopal *et al.*, 1998). Display of the mere anchoring domain multiplied the metallosorption capability of *S. cerevisiae* from solutions containing 100 μM metal twofold for Cd^{2+} (to 19.5 ± 2.3 nmol Cd^{2+} per mg dry cell weight) and Zn^{2+} (to 14.3 ± 1.6 nmol Zn^{2+} per mg dry cell weight) and fourfold for Cu^{2+} (to 25.2 ± 1.8 nmol Cu^{2+} per mg dry cell weight). Surface display of CP2 additionally increased Cd^{2+} sorption by 30% and HP3 display increased capability of the engineered strain to adsorb Zn^{2+} by 20%. Display of NP peptide specifically increased amount of sorbed Pb^{2+} by 22% to 61.5 ± 1.0 nmol per mg dry cell weight. Purified fusions of HP3 to *E. coli* proteins allowed us to demonstrate that HP3 is effective in Zn^{2+} sequestration forming 3 Zn^{2+} binding sites with an apparent dissociation constant of 1.2×10^{-7}. Accordingly, the increased amount of Zn^{2+} sorbed by yeasts due to HP3 display was equivalent to 3.7 Zn^{2+} atoms per displayed HP3 peptide. Our data thus indicate that overproduction of natural mannoproteins leading to their surface exposure is a promising approach to improve the metallosorption properties of yeast biosorbent, while display of a Cd^{2+}-specific CP, Zn^{2+}-specific or Pb^{2+}-specific peptides forming high-affinity metal coordination spheres would not favor metal displacement over other yeast cell wall components and thus would contribute less to the overall metallosorption properties of the modified biosorbent

Funded by grants of Czech Ministry of Education no. 1M6837805002 and MSM 6046137305.

Sousa, C., Kotrba, P., Ruml, T., Cebolla, A. and De Lorenzo, V. (1998) Metalloadsorption by *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB. J. Bacteriol. 180, 2280-2284.

Vinopal S., Ruml, T. and Kotrba, P. (2007) Biosorption of Cd^{2+} and Zn^{2+} by cell surface-engineered *Saccharomyces cerevisiae*. Int. Biodeter. Biodegr. 60,96-102.

25. Production of recombinant mGM-CSF in glycoengineered *P. pastoris*

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http://www.dmbr.ugent.be

Current manufacturing methods of heterologous proteins based on mammalian cell culture do not allow for the control of glycosylation and produce a mixture of different glycoforms, which matter of fact hampers the optimisation of a drug's therapeutic profile. Since glycoengineered *Pichia pastoris* strains are capable of processing proteins with N-glycans similar to that of higher mammals and humans, their potential for manufacturing fully functional therapeutic glycoproteins is now being examined. As a suitable model, expression of the recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was studied systematically using a Mut^+ strain engineered to secrete proteins with a homogeneous $Man_5GlcNAc_2$ N-glycan profile. In a series of experiments the uncoupled effect of specific growth rate and growth temperature on (1) product formation, (2) efficiency and (3) homogeneity of N-glycosylation was quantified. Continuous culture experiments served as means to unravel the mechanisms that underlie the expression of correctly processed rmGM-CSF protein. The knowledge gained on the relationships between culture conditions and product expression (both in terms of quantity and quality) was used to design an optimum manufacturing procedure in fedbatch mode.

For cells grown in mineral medium at 20°C, neither the product yield nor the productivity were significantly affected by the specific growth rate, *i.e.* dilution rate of continuous culture was set at 45% and 71% of μ_{max}. At 30°C and 45% of μ_{max}, a product yield of (4.78 ± 0.42) mg product per g cell dry weight was found to be more than twice as high as those achieved at 20°C or 30°C and 71% μ_{max}. However, productivity remained unaffected at the two dilution rates studied at 30°C. Regardless of the cultivation mode (*i.e.* continuous, fedbatch), product size ranged between 14 and 25 kDa depending on the particular glycosylation attached. DSA-FACE analysis of N-glycan profiles in culture supernatants revealed a gradually increasing heterogeneity as the continuous cultivation proceeded, with the highest observed at 20°C and 71% μ_{max}. Hence, the most favourable conditions enhancing the glycans' homogeneity are temperatures close to growth optimum and low specific growth rates.

So far, the best production was attained in a high-cell-density (HCD) fedbatch culture at 30°C, *i.e.* 370 mg/l of rmGM-CSF with >80% homogenous $Man_5GlcNAc_2$ structures. Due to the linear substrate addition during 55 hours of production (induction) phase, the specific growth rate was constant within the first 28 hours of production at 19% of μ_{max} (*i.e.* 0.02 h^{-1}) with methanol as the sole source of carbon and energy. The product expression was triggered after a temporary accumulation of methanol (up to 9 g/l) and clearly exhibited growth-associated kinetics with a yield of (8.65 ± 1.89) mg/g and the highest product formation rate of (0.20 ± 0.06) mg/(g h). Provided the high product yield observed in the first production phase could be maintained during the whole HCD-fedbatch, final titres with a magnitude of several g/l are anticipated for this costly product.

These phenomena open an interesting range of possible future investigations on processing N-glycans using glycoengineered *Pichia* cultures grown with different carbon sources and/or under the limitations of elements other than carbon.

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26. Fast gene copy number determination in the methylotrophic yeast, *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* is a well known expression system. Since no stable plasmids have been reported so far for this task, genes are usually integrated into the genome mostly the *AOX1* locus. Using auxotrophic markers the number of integrated expression cassettes is difficult to manipulate. Antibiotic resistance genes can offer some relief and multi copy integration can help to construct highly productive strains. On the other hand, strain engineering, promoter studies, as well as co-expression of helper proteins to mention some examples, where the knowledge of the expression cassette copy number is especially important, since comparison of strains with unknown number of integrated expression cassettes can yield in false interpretations of experimental results (Inan *et al*., 2006; Hartner). The most common used method for copy number determination is Southern analysis. With the appropriate choice of restriction enzyme and probe sequence, the number of bands or their intensity should allow to determine the copy number in the genome (Bubner *et al*., 2004). Although Southern analysis is reliable, it is time-consuming and laborious and requires large amounts of genomic DNA. Furthermore, in the case of restriction site loss or concatemers the number of bands does not correspond to the copy number (Bubner *et al*., 2004; Ballester *et al*., 2004). Overall, this is not a very sensitive and reliable way to determine the expression level (Bubner *et al*., 2004). These reasons lead us to look for an alternative, a more simple and quick method for copy number determination in *P. pastoris* genomic DNA. Real time PCR has been established for copy number determination of expression cassettes integrated in the P. pastoris genome. Quantitative PCR emerged to an important, widely used tool for many scientific applications like measuring mRNA levels, DNA copy number, allelic discrimination and measuring viral titers (Ginzinger 2002). Careful consideration of the assay design, template preparation and analytical methods were essential for accurate gene quantitative amplification (Ginzinger 2002). Two different detection methods, SYBR Green as well as TaqMan strategies have been tested. The establishment of a suitable reference system for the correct evaluation of the experimental results, as well as the impact of two different calculation methods will be reported.

Inan, M., Aryasomayajula, D., Sinha, J. and Meagher, M.M. (2006) Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. Biotechnol. Bioeng. 93, 771-778.

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27. Process development for the production of 3-DMC- an anti-cancer drug at commercial scale

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Colchicine (a plant alkaloid) derivatives *i.e.* 3-demethylatedcolchicine (3-DMC), colchicoside and thio-colchicoside are proven to be an effective anti-tumor drug, and in future these natural products will have good commercial demand. Presently, these derivatives could produce by chemical process but due to the unspecific reaction during conversion process and problem during separation of derivatives, increase the cost of product. So, the practiced process hindered to commercialize the colchicine derivatives. In order to commercialize these compounds, to find out an effective microbe which could able to bring regiospecific changes in the colchicine at C-3 position of ring A. We described a novel mutant of *Bacillus megaterium* ACBT03 having potential of about 73% conversion of colchicine to its respective 3-demetyl derivatives was used for scale up process at 70 l fermenter. Besides this, it was targeted to standardized, the downstream process to recover about 70% 3-demethylated colchicine having 99% purity.

28. Establishment of a novel system for the detection of spoilage microorganisms in wine

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Since about 15 years one observes a global climate change that is characterized by higher temperatures, greater differences in temperatures and monsoon-type precipitation. Amounts of precipitation in single months are partly far below the long-term average. Viticulture regions which have been among the “cool climate” regions in the past have changed in large part to “hot climate” regions. This climate change does not only have effects on viticulture in terms of grape production (limitation of quantity, irrigation), but also affects the composition of the microbial community on grapes and during vinification.

To assure high quality of wine, it is essential to early detect spoiling yeasts and bacteria during wine production. *Brettanomyces bruxellensis* causes off-flavours described as “horse-sweat” or “medicinal note”, *Pediococcus spp.* cause formation of increased amounts of biogenic amines, diacetyl and “lindton”, and *Lactobacillus spp.* can cause the formation of acetic acid, biogenic amines and “mousy taint”. Additionally, both *Pediococcus spp.* and *Lactobacillus spp.* are able to produce lactic acid (mainly D-lactic acid) if residual sugar is present.

We established a specific and sensitive system based on real-time PCR in order to early detect these microorganisms at species level qualitatively and quantitatively and before the formation of undesirable metabolites occurs. The results are compared with chemical analytical as well as sensorial data.

29. The method of image analysis as new tool for monitoring of *Pichia* cell aggregation

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The method of image analysis has been developed recently but immediately has become routine in several cell technology applications. For many decades the microscopic specimens were counted or measured manually. The method of image analysis enables automation for such measurements now. The basic image analysis system consists of video or digital camera, microscope and software for subsequent measurement. The ability of cell aggregation can be observed in all taxa of microorganisms (bacteria, yeast, fungi etc.). Although the process of aggregation has been widely studied it remains still poorly understood. A lot of factors (genetic, physiological and nutritional) determine the ability and rate of cluster forming. The yeast *Pichia* belongs to methylotrophic yeast. It can consume methanol as a sole carbon source where glucose or glycerol is not present in medium. Recombinant *Pichia pastoris* is used for production of large quantities of specialized proteins. To metabolize methanol *Pichia pastoris* has an alcohol oxidase (*AOX*) gene. The *P. pastoris* expression system uses the methanol-induced alcohol oxidase promoter (*AOX1*) which controls the gene that codes for the expression of alcohol oxidase (the enzyme which catalyzes the fist step in the metabolism of methanol). Using genetic techniques genes for pharmaceutical proteins can be inserted next to the *AOX*-gene in *Pichia* allowing the yeast to produce the protein. The aim of the work is to find out the method for monitoring of cell clusters during cultivation process. *Pichia anomala* (DBM 2111) and two recombinant strains of *Pichia pastoris* were chosen for our experiments. Yeasts were cultivated on complex medium, glycerol, glucose or methanol was used as a sole carbon sources. During experiments the ability of cell cluster forming in shake flasks and bioreactor (Bioreactor BIOSTAT, B. Braun Biotech International, Melsungen, Germany) was monitored and compared. The microscopic observation and later image analysis were proposed for monitoring of cell cluster forming.

30. Engineering of plant vacuolar proteins for production in *Pichia pastoris*

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Protein targeting to plant vacuoles is not yet fully understood due to complexity of the signals and their low amino acid sequence homology (Carter *et al*., 2004). When producing such proteins in a recombinant yeast system, problems may arise from misinterpretation of the signal sequence by the heterologous host (Jolliffe *et al*., 2003). We study the family of seven cytokinin dehydrogenase enzymes (EC 1.5.99.12) that control the metabolism of plant hormones cytokinins in *Arabidopsis thaliana*. Individual members of the family share conserved protein regions of high homology (e.g. FAD-binding domain), but differ in catalytic properties, subcellular localization and expression pattern in plant organs and tissues (Werner *et al*., 2003). Four of the enzymes are targeted to the plant secretory pathway, two to vacuoles and one is presumably localized to cytosol.

Two genes coding for vacuolar cytokinin dehydrogenase enzymes AtCKX1 and AtCKX3 from *Arabidopsis thaliana* were chosen for protein overproduction in *Pichia pastoris*. Previous experiments indicated that polyhistidine fusion to the C-terminus of the recombinant cytokinin dehydrogenase interferes with the structure of the active site and prevents the protein from proper folding. Therefore a novel vector *pGAPZαA* was prepared from *pGAPZα* by inserting a coding sequence for 10x His from *pET16b* between the α-factor coding sequence and the multiple cloning site. With both studied proteins, the sequence coding for N-terminal fragment predicted by SignalP 3.0 as a leader peptide was removed before cloning into *pGAPZαA*. Recombinant proteins to be obtained using the modified vector were designed for secretion from yeast using the N-terminal α-factor signal sequence and for binding to Ni-NTA columns thanks to the N-terminal polyhistidine tag.

Initial experiments, however, did not lead to secretion of active proteins. More accurate amino acid sequence analysis of AtCKX1 and AtCKX3 revealed the presence of a region weakly resembling N-terminal sequence-specific vacuolar sorting signal (ssVSS) that typically contains a degenerate signal [N/L]-[P/I/L]-[I/P]-[R/N/S], also called NPIR consensus sequence, and targets proteins to lytic vacuoles after pre-processing in the endoplasmic reticulum (Matsuoka and Nakamura, 1999). Only after deleting this motif, active plant vacuolar cytokinin dehydrogenases were obtained in *Pichia pastoris*.

This work was supported in part by the grant MSM 6198959216 from the Ministry of Education, Youth and Sports, Czech Republic.

Carter, C., Pan, S., Zouhar, J., Avila, E.L., Girke, T. and Raikhel, N.V. (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16, 3285-3303.

Jolliffe, N.A., Ceriotti, A., Frigerio, L. and Roberts, L.M. (2003) The position of the proricin vacuolar targeting signal is functionally important. Plant Mol. Biol. 51, 631–641.

Matsuoka, K. and Nakamura, K. (1999) Large alkyl side-chains of isoleucine and leucine in the NPIRL region constitute the core of the vacuolar sorting determinant of sporamin precursor. Plant Mol. Biol. 41, 825–835.

Werner, T., Motyka, V., Laucou, V., Smets, R., van Onckelen, H. and Schmülling, T. (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532-2550.

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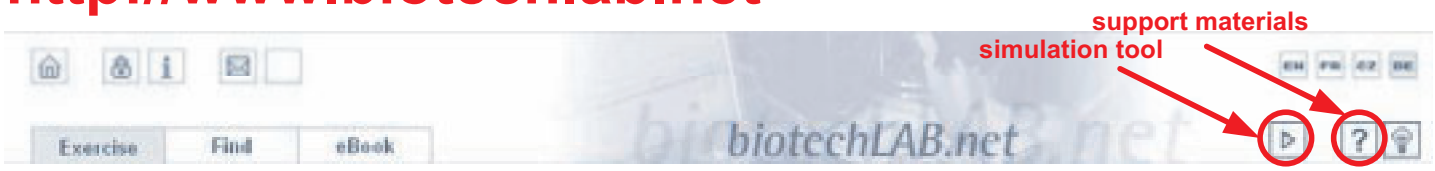

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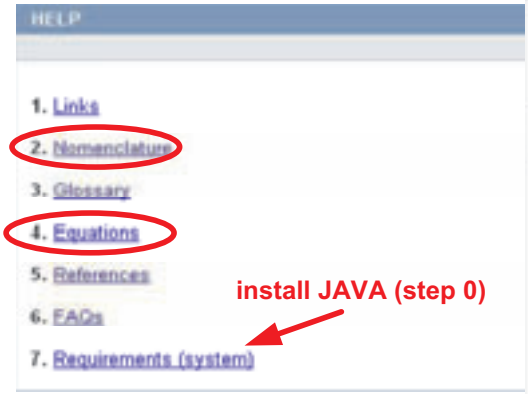

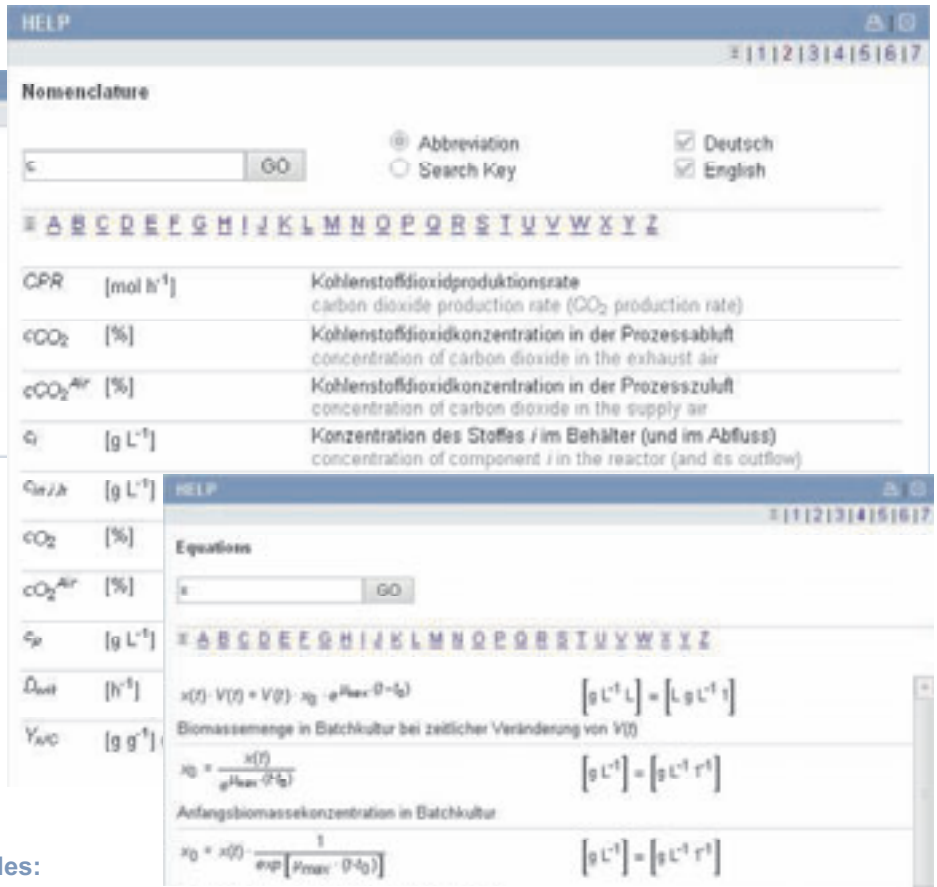

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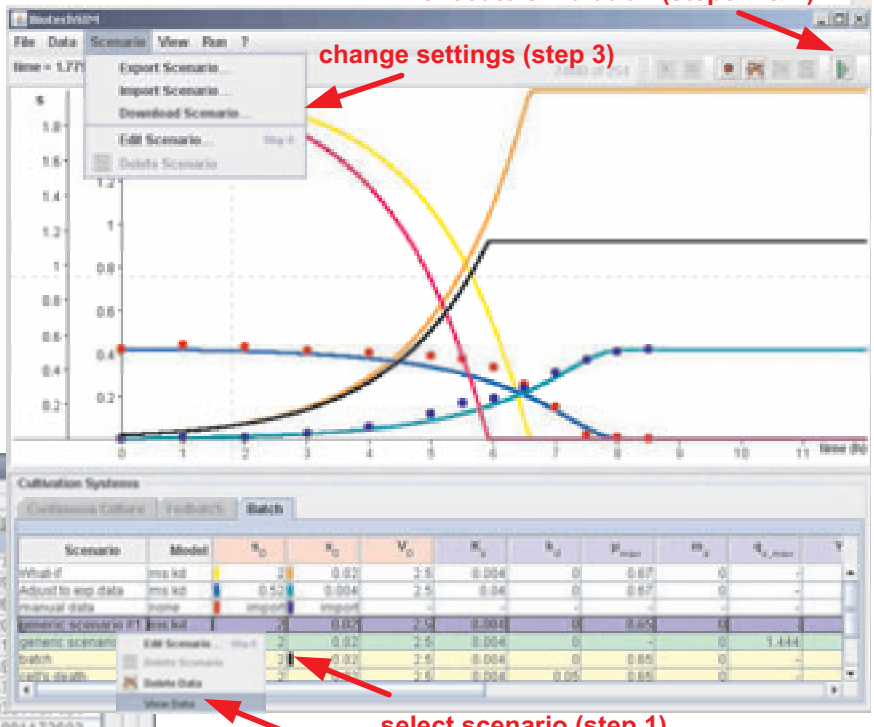

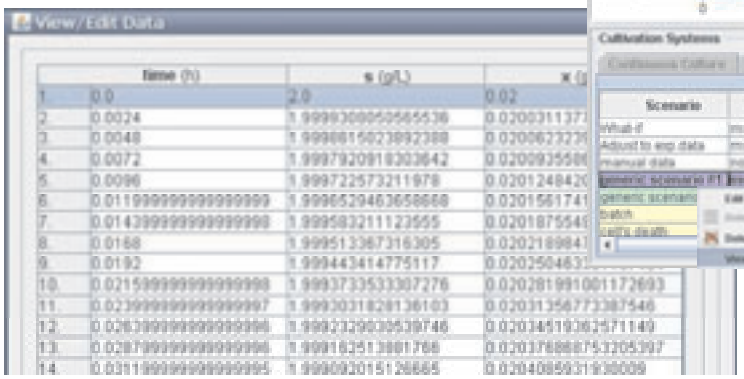

Thursday 22 May 2008 — Morning

High-Level Protein Expression

08:30–09:00	Registration (Campus Grüental)
	Chairperson: Anton Glieder Co-chairperson: Verena Looser
09:00–09:30	Opening session
09:30–10:00	**A comparative view on yeast expression hosts *S. cerevisiae* and *P. pastoris*** Christine Lang, TU Berlin D
10:00–10:10	Discussion
10:10–10:40	Coffee break & poster session and exhibition
10:40–11:10	**Fungal cell factories: production and screening of antibody (-fragments)** Peter J. Punt, TNO, AJ Zeist NL
11:10–11:20	Discussion
11:20–11:50	**Intracellular expression of heterologous gene encoding bacterial penicillin acylase in *Pichia pastoris*** Pavel Kyslík, Institute of Microbiology ASCR, Prague CZ
11:50–12:00	Discussion
12:00–12:40	**Genomics comes to the recombinant protein production yeast *Pichia pastoris*** James M. Cregg, Keck Graduate Institute of Applied Life Sciences, Claremont USA
12:40–12:50	Discussion
12:50–14:00	Lunch & poster session and exhibition

Thursday 22 May 2008 — Afternoon

Process Engineering vs. Strain Design

	Chairperson: Leona Paulová Co-chairperson: Christian Meier
14:00–14:30	**Alternative cultivation strategies with *Pichia pastoris* for the production of therapeutic recombinant proteins** Carmen Jungo, Lonza Ltd, Visp CH
14:30–14:40	Discussion
14:40–15:10	**Production of cytochrome P450-metabolites using recombinant fission yeast *Schizosaccharomyces pombe*** Matthias Bureik, PomBioTech GmbH, Saarbrücken D
15:10–15:20	Discussion
15:20–15:50	Coffee break & poster session and exhibition
15:50–16:20	**Methanol free protein expression by *Pichia pastoris* using methanol inducible promoter variants** Claudia Ruth, Institute of Molecular Biotechnology, TU Graz A
16:20–16:30	Discussion
16:30–17:00	**Strain design and its impact on process development in *H. polymorpha* and other yeasts** Gerd Gellissen, PharmedArtis GmbH, Aachen D
17:00–17:10	Discussion
17:10–17:40	**Poster awards**
17:40–19:00	Aperitif & poster session and exhibition
19:00– open end	Gala dinner (Campus Reidbach) Addresses by: Václav Paces, President of ASCR H.E. Boris Lazar, Czech Ambassador to Switzerland Ernst Stocker, Mayor of Wädenswil

Friday 23 May 2008 — Morning

Postranslational Modifications

08:30–09:00	Registration (Campus Grüental)
	Chairperson: Nico Callewaert Co-chairperson: Sandra Núñez
09:00–09:30	**N-linked protein glycosylation in yeast** Markus Aebi, Swiss Federal Institute of Technology (ETH), Zurich CH
09:30–09:40	Discussion
09:40–10:10	**Secretory system engineered yeast expression systems: taking the prototypes further** Nico Callewaert, Flanders Institute for Biotechnology vzw, Ghent B
10:10–10:20	Discussion
10:20–10:50	Coffee break & poster session and exhibition
10:50–11:20	***Yarrowia lipolytica:* a versatile expression system for the production of biopharmaceuticals** Adriana Botes, Oxyrane UK Ltd, Manchester UK
11:20–11:30	Discussion
11:30–12:00	**Modified substrates in enzymatic glycosylations: a simple route to complex saccharides** Pavla Bojarová, Institute of Microbiology ASCR, Prague CZ
12:00–12:10	Discussion
12:10–12:50	**Panel discussion**
12:50–14:00	Lunch & poster session and exhibition

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Friday 23 May 2008 — Afternoon

Further Opportunities

	Chairperson: Hans-Peter Meyer Co-chairperson: Radka Strnadová
14:00–14:30	**Physiological characterisation and strain engineering of *Pichia pastoris* by transcriptomics and proteomics** Brigitte Gasser, BOKU-University of Natural Resources and Applied Life Sciences, Vienna A
14:30–14:40	Discussion
14:40–15:10	***Pichia pastoris:* a powerful expression system to support drug discovery projects in pharmaceutical industry** Ralf Thoma, F. Hoffmann-La Roche AG, Basel CH
15:10–15:20	Discussion
15:20–15:50	Coffee break & poster session and exhibition
15:50–16:20	**Enzymatic synthesis of glycoconjugates** Oreste Ghisalba, Novartis Pharma AG, Basel CH
16:20–16:30	Discussion
16:30–17:00	**Co-expression of homologous and heterologous genes as tools for advancement of established and new yeast expression systems** Adelheid Degelmann, Artes Biotechnology GmbH, Langenfeld D
17:00–17:10	Discussion
17:10–17:40	**Complex mammalian proteins easily produced in *Pichia pastoris*: the case of vascular endothelial growth factors** Kurt Ballmer-Hofer, Paul Scherrer Institute, Villigen CH
17:40–17:50	Discussion
19:00– open end	Alumni get-together