Pichia 2009

Tucson, Arizona
Marriott Starr Pass Resort & Spa

We appreciate our sponsors:

Research Corporation Technologies

Lonza

Table of Contents

Resort and Conference Map 3

Schedule at a Glance 4

Detailed program 6

Monday, October 19, 2009

Keynote 6

Session 1: Genomic Insights 6

Session 2: Optimization of Strains and Fermentation 7

Session 3: Posters 7

Tuesday, October 20, 2009

Session 4: Glycoengineering and Novel Applications 8

Session 5: Products and Production 9

Wednesday, October 21, 2009

Session 6: Production and Products in *Pichia* 9

Poster Session – List of Posters 11

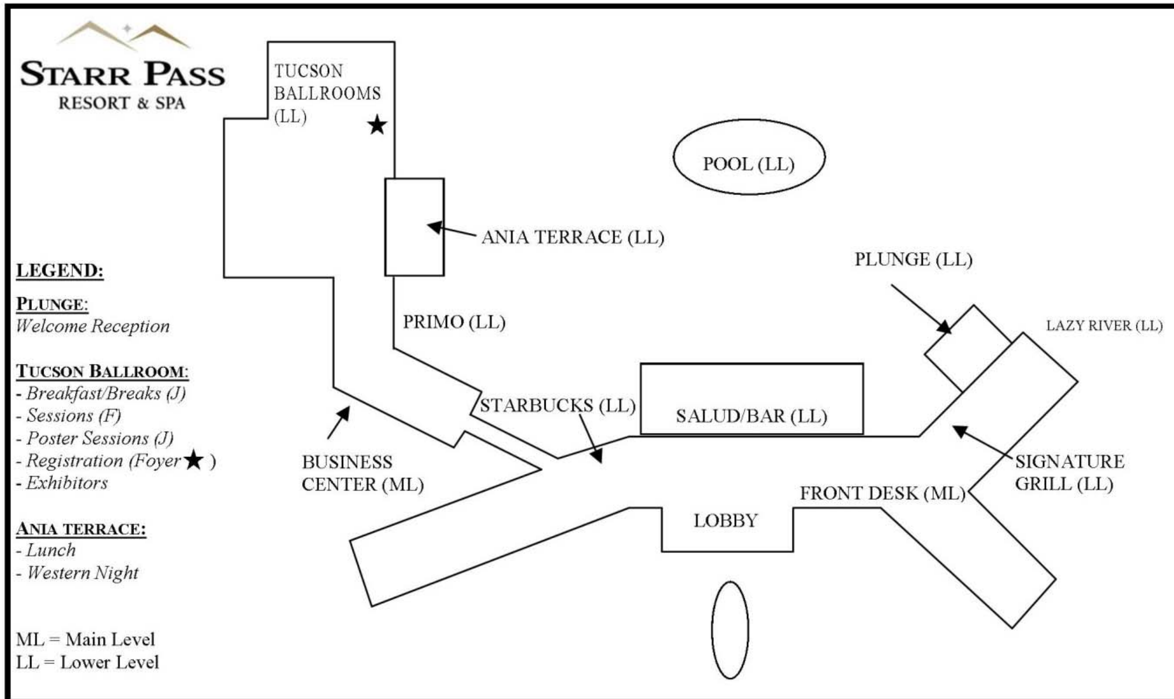
Abstracts 15

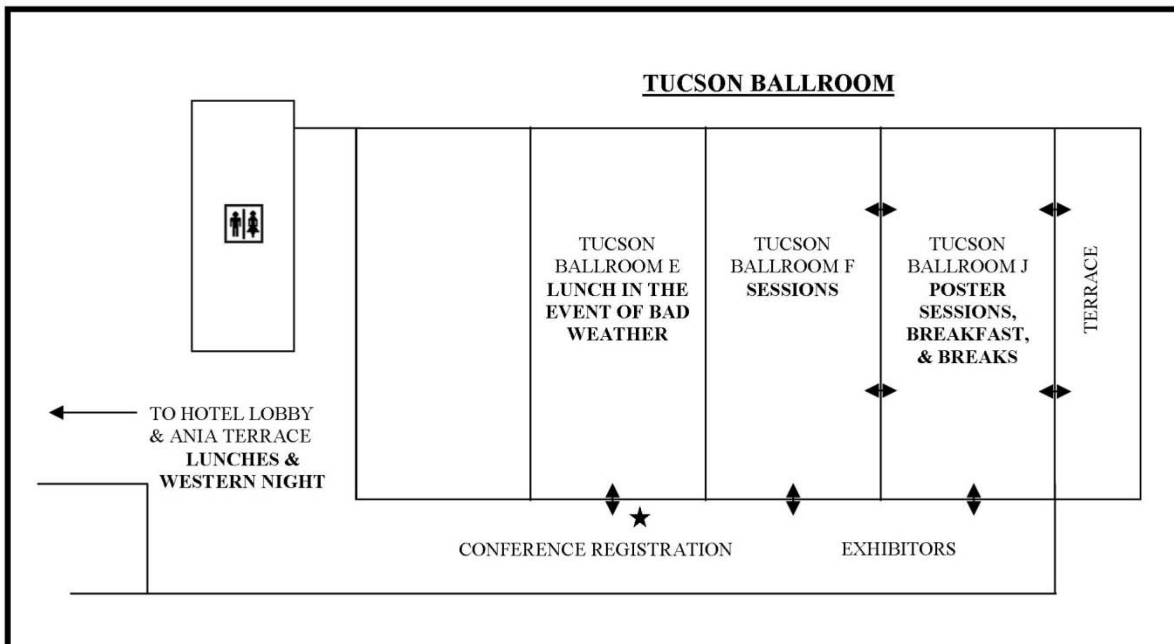
Oral Presentations 15

Poster Presentations 43

Attendee List 62

Resort and Conference Map





RESORT HOURS OF OPERATION:

Starbucks: *5am—5pm*
Signature Grill: *Breakfast 6:30am—11am*
Lunch 11am—3pm
Bar 3pm—5:30pm
Dinner 5:30pm—10pm
In-room Dining: *6am—11pm*
Salud: *3pm—Late Daily (toast at 5:30pm)*
Golf Pro Shop: *6am—6pm*
Water Collection: *pools 6am—11pm*
lazy river 11am—6pm

Marketplace: *7am—10pm*
Hashani Spa: *8am—5pm*
Fitness Center: *24 hours*
Business Center: *M-F 8am—6pm*
Sat. 9am—4pm

Updated October 8, 2009—Subject to Change

Schedule at a Glance

Sunday, October 18, 2009

1:30pm – 5:00pm	Conference Registration	Resort Main Lobby
4:30pm – 7:30pm	Welcome Reception	Plunge

Monday, October 19, 2009

6:30am – 5:00pm	Conference Registration	Tucson Ballroom Foyer
7:30am – 8:30am	Continental Breakfast	Tucson Ballroom J
Morning Session:		Tucson Ballroom F
7:30am – 8:00am	AV check for today's speakers	
8:30am	Welcome	
8:35am	Keynote Lecture	
9:15am	Session 1: Genomic Insights	
10:00am	Morning Coffee and Poster Set-up	Tucson Ballroom J
10:30am	Session 1 continues	Tucson Ballroom F
Noon	Lunch	Ania Terrace
Afternoon Sessions:		Tucson Ballroom F
1:15pm	Session 2: Optimization of Strains and Fermentation	
3:00pm	Afternoon Coffee	Tucson Ballroom J
3:25pm	Session 2 continues	Tucson Ballroom F
4:20pm	Session ends	
5:00pm – 8:00pm	Session 3: Posters and cash bar	Tucson Ballroom J

Tuesday, October 20, 2009

7:30am – 3:00pm	Conference Hospitality Desk Open	Tucson Ballroom Foyer
7:30am – 8:30am	Continental Breakfast	Tucson Ballroom J

Morning Session: Tucson Ballroom F

7:30am – 8:00am	AV check for today's speakers	
8:30am	Session 4: Glycoengineering and Novel Applications	
10:20am	Morning Coffee	Tucson Ballroom J
10:40am	Session 4 continues	Tucson Ballroom F
11:45am	Lunch	Ania Terrace

Afternoon Sessions: Tucson Ballroom F

1:00pm	Session 5: Products and Production	
3:15pm	End of session and Poster removal	Tucson Ballroom J
3:15pm	Free Time	
6:00pm	Western Evening	Ania Terrace

Wednesday, October 21, 2009

7:30am – 11:00am	Conference Hospitality Desk Open	Tucson Ballroom Foyer
7:30am – 8:30am	Continental Breakfast	Tucson Ballroom J

Morning Session: Tucson Ballroom F

7:30am – 8:00am	AV check for today's speakers	
8:30am	Session 6: Production and Products in *Pichia*	
10:10am	Morning Coffee	Tucson Ballroom J
10:30am	Session 6 continues	Tucson Ballroom F
11:15am	Closing Remarks	
11:20am	Conference close. Pick up lunch	Tucson Ballroom Foyer

Detailed Program

Sunday, October 18, 2009

1:30pm – 5:00pm	Conference Registration	Resort Main Lobby
4:30pm – 7:30pm	Welcome Reception	Plunge

Monday, October 19, 2009

6:30am – 5:00pm	Conference Registration	Tucson Ballroom Foyer
7:30am – 8:30am	Continental Breakfast	Tucson Ballroom J/Terrace

Morning Session — Tucson Ballroom F

8:30am **Welcome and Housekeeping Announcements**
David Bramhill, RCT

8:35am **Keynote Lecture**
T1: *Pichia:* A historic perspective with a look to the future
James Cregg
Keck Graduate Institute, CA

9:15am **Session 1: Genomic Insights**
Chair: Nico Callewaert

9:20am **T2: Entering the genomic era:**
The *Pichia pastoris* genomic sequence and the information within
Kristof De Schutter
VIB and Ghent University, Belgium

10:00am **Morning Coffee and Poster Set-up** — Tucson Ballroom J

10:30am **Session 1 continues** — Tucson Ballroom F

T3: New tools for accelerated protein expression and engineering using *Pichia pastoris*
Anton Glieder
Graz University of Technology, Austria

11:15am **T4: Identification of hypoxia induced promoters in *Pichia pastoris***
Andrea Camattari
Bioprocessing Technology Institute, Singapore

11:35am	**T5: Going double-digit with *Pichia*: high-level expression of human serum albumin and transferrin as well as fusion proteins driven by AOX1 promoter library** Roland Weis VTU Technology, Austria	
Noon	**Lunch**	Ania Terrace

Afternoon Sessions Tucson Ballroom F

1:15pm	<u>**Session 2: Optimization of Strains and Fermentation**</u> Chair: Karin Kovar	
	T6: *Pichia* Fermentation: Past, present and future. Michael Meagher University of Nebraska, Lincoln, NE	
1:45pm	**T7: *Pichia* technology at the cutting edge: process development meets strain design** Karin Kovar Institute of Biotechnology, Zurich University of Applied Sciences (ZHAW)	
2:15pm	**T8: Single-cell analysis of protein expression and secretion in *Pichia pastoris* by microengraving** Kerry Routenberg Love Department of Chemical Engineering, Massachusetts Institute of Technology	
3:00pm	**Afternoon Coffee**	Tucson Ballroom J
3:25pm	**Session 2 continues**	Tucson Ballroom F
	T9: A scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime Richard A.J. Darby Aston University, UK	
3:45pm	**T10: On the way to a self optimizing fully automated and global observable integrated bioplant for production of recombinant proteins with *Pichia pastoris*** Reiner Luttmann Hamburg University of Applied Sciences, Germany	
4:20pm	**Session ends**	
5:00pm – 8:00pm	<u>**Session 3**</u> Poster Sessions, Reception and Cash Bar	Tucson Ballroom J

Tuesday, October 20, 2009

7:30am – 3:00pm	Conference Hospitality Desk Open	Tucson Ballroom Foyer
7:30am – 8:30am	Continental Breakfast	Tucson Ballroom J / Terrace
Morning Session		Tucson Ballroom F
8:30am	**Session 4: Glycoengineering and Novel Applications** Chair: Roland Contreras	
8:35am	**T11: Protein N-Glycan engineering in *Pichia pastoris* for increased homogeneity: an introduction and early results** Roland Contreras Ghent University and Flanders Institute for Biotechnology, Belgium	
9:00am	**T12: Engineering complex-type N-glycosylation in *Pichia pastoris* using *Pichia* GlycoSwitch™ technology** Nico Callewaert Flanders Institute for Biotechnology (VIB) and Ghent University, Belgium	
9:45am	**T13: Expression of variable and invariable trypanosomal glycoproteins in *Pichia pastoris* for diagnosis of African trypanosomiasis and surra** Stijn Roge Institute for Tropical Medicine, Antwerp, Belgium	
10:05am	**T14: Difficult proteins: expression and secretion in *Pichia pastoris* using novel sumo system** Raymond Peroutka LifeSensors, Inc., Malvern, PA	
10:20am	**Morning Coffee**	Tucson Ballroom J
10:40am	**Session 4 continues**	Tucson Ballroom F
	T15: Expression of membrane-bound monoamine oxidases from three different organisms in *Pichia pastoris* Milagros Aldeco Department of Biochemistry, Emory University School of Medicine, GA	
11:00am	**T16: Transcriptomics-based engineering of *Pichia pastoris* for improved production of eukaryotic membrane proteins** Maria Freigassner Graz University of Technology & K+ Research Centre, Graz, Austria	
11:20am	**T17: Expanding the genetic repertoire of *Pichia pastoris*** Travis Young Scripps Research Institute, La Jolla, CA	
11:45am	**Lunch**	Ania Terrace

Afternoon Sessions Tucson Ballroom F

1:00pm **Session 5: Products and Production**
Chair: Kerry Chester

1:05pm **T18: Bench to bedside with *Pichia:* Recombinant antibody-based cancer treatments**
Kerry Chester
University College London, UK

1:45pm **T19: Discovery and development of Ecallantide**
Mark DeSouza
Dyax Corp., MA

2:25pm **T20: Manufacturing of proteins in *Pichia* - present and future**
Gesche Bernhard
Lonza Ltd., Visp, Switzerland

2:55pm **T21: *Pichia pastoris* whole cell biocatalyst**
Sandra Abad
Research Centre Applied Biocatalysis, Graz, Austria

3:15pm **End of session and Poster removal** Tucson Ballroom J

3:15pm **Time on own**

6:00pm **Western Evening** Ania Terrace

Wednesday, October 21, 2009

7:30am – 11:00am Conference Hospitality Desk Open Tucson Ballroom Foyer

7:30am – 8:30am Continental Breakfast Tucson Ballroom J

Morning Session Tucson Ballroom F

8:30am **Session 6: Production and Products in *Pichia***
Chair: Mark DeSouza

T22: The Glycoprotein hormones, receptors and *Pichia pastoris*
Rajan R. Dighe
Indian Institute of Science, Bangalore, India

9:15am **T23: Characterization and scale up of *Pichia pastoris* processes for biopharmaceutical production**
John Liddell
Avecia Biologics, Teeside, UK

9:45am	**T24: Development of an automated cultivation process for the expression of recombinant house dust mite allergen Der p 1 in *Pichia pastoris*** Gesine Cornelissen Hamburg University of Applied Sciences, Germany	
10:10am	**Morning Coffee**	Tucson Ballroom J
10:30am	**Session 6 continues**	Tucson Ballroom F
	T25: Production of wasp venom allergens in *Pichia pastoris* Irina Borodina Center for Microbial Biotechnology, Technical University of Denmark	
10:50am	**T26: Evaluation of methylotrophic yeasts as efficient expression hosts for the production of a glycoprotein for vaccine application; pastoris or angusta; what's the difference?** Geert Deschamps Innogenetics Biologicals, Belgium	
11:15am	**Closing Remarks** David Bramhill RCT, Tucson, AZ	
11:20am	**Conference close. Pick up lunch**	Tucson Ballroom Foyer

Poster Session

P1 **Characterization of house dust mite allergen Der p 1 expressed in *Pichia pastoris***

A Nandy, G. Cornelissen, M. Wald, H. Kahlert, O. Cromwell, H. Fiebig
Allergopharma Joachim Ganzer KG
Reinbek, Germany

P2 **Continuous, on-line monitoring of glycerol and methanol during *Pichia pastoris* fermentations using near infrared spectroscopy**

Elizabeth R. Gibson[1], Jonathon T. Olesberg[1,2], Mark A. Arnold[1,2]
[1]*ASL Analytical, Inc, Coralville, IA*
[2]*Optical Science and Technology Center and Department of Chemistry, University of Iowa, Iowa City, IA*

P3. **Bioprocess scale-up and optimisation of erythropoietin production in *Pichia pastoris***

Nagamani Bora and Roslyn Bill.
Aston University
Birmingham, UK.

P4 **Antifoams in Fermentation Processes**

S J Routledge and R M Bill
Aston University
Birmingham, UK.

P5 **Prolipase Signal Sequence Stabilizes the Expression of Bovine Trypsinogen in *Pichia pastoris***

Nagaraj Govindappa, Nandini Nataraj, Reena Darshan, Sanjay Tiwari, Partha Hazra, Mukesh Patale, Pritish Sarkar, Gokul Jothiraman and Kedarnath N Sastry
Biocon Limited
Bangalore, India.

P6 **Methanol free, inducible expression of porcine trypsinogen employing new promoter variants in *Pichia pastoris***

Ruth, C.[1], Zuellig, T.[3], Mellitzer, A.[1], Looser, V. [3], Kovar, K.[3] and Glieder, A.[1,2]
[1] *Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Research Centre Applied Biocatalysis, Graz, Austria*
[3] *Institute of Biotechnology, University of Applied Sciences Zürich*
Waedenswil, Switzerland

P7 Sequencing and Annotating *Pichia pastoris* CBS 7435

Andreas Küberl[1,2], Gerhard Thallinger[1], Helmut Schwab[3], Harald Pichler[3]
[1] Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria
[2] Research Centre Applied Biocatalysis, Graz, Austria
[3] Institute for Genomics and Bioinformatics, Graz University of Technology
Graz, Austria

P8 Advancements in *Pichia pastoris* expression platform: improving targeting efficiency for the introduction of new auxotrophies

Laura Näätsaari[1], Beate Pscheidt[2], Claudia Ruth[1], Sandra Abad[2], Kerstin Kitz[2], Stefan Ertl[1], Clemens Mayer[1], Viktorija Vidimce[1], Roland Weis[3], Anton Glieder[1]
[1]Department of Molecular Biotechnology, Graz University of Technology, Austria
[2]Research Centre Applied Biocatalysis, Graz, Austria
[3]VTU Technology, Grambach, Austria

P9 Expression and characterization of cytochrome P450 2D6 Variants in *Pichia pastoris*

Geier, M.[1], Marienhagen, J.[2], Schwaneberg, U.[2], and Glieder, A.[1]
[1]Institute of Molecular Biotechnology, Graz University, Graz, Austria
[2]Department of Biotechnology, RWT, Aachen, Germany

P10 An engineering approach for online monitoring of dynamics in protein expression of *Pichia pastoris* based on PAT-Applications and mathematical process description

R. Luttman, L. Hukelmann, K. Logering, A. Kazemi, G. Cornelissen
Research Center of Bioprocess Engineering and Analytical Techniques HAW-Hamburg University of Applied Sciences, Hamburg, Germany

P11 Production and purification of artificial malaria vaccines from high cell density cultivations with *Pichia pastoris*

S. Martens[1], B. Faber[2], S.-O. Borchert[1], P. Hartwich[1], G. Cornelissen[1], B. Anspach[1], R. Luttmann[1]
[1] Research Center of Bioprocess Engineering and Analytical Techniques
HAW – Hamburg University of Applied Sciences, Hamburg, Germany
[2] Biomedical Primate Research Center, Department of Parasitology
Rijswijk,The Netherlands

P12 Optimization of recombinant enzyme production with *Pichia pastoris* in a fully automated integrated scale down production plant

K. Lögering, J. Fricke, C. Müller, H.-P. Bertelsen, U. Scheffler, R. Luttmann
Research Center of Bioprocess Engineering and Analytical Techniques
HAW – Hamburg University of Applied Sciences, Hamburg, Germany

P13 Conception of a sequential/parallel DoE-design of experiments in a multifermenter system BIOSTAT® Q*plus*6 for optimal recombinant malaria vaccine production

J. Fricke[1], F. Tatge[1], H.-P. Bertelsen[1], U. Scheffler[1], B. Faber[2], R. Luttmann[1]
[1] Research Center of Bioprocess Engineering and Analytical Techniques
HAW – Hamburg University of Applied Sciences, Hamburg, Germany
[2] Biomedical Primate Research Center, Department of Parasitology,
Rijswijk, The Netherlands

P14 Expression and characterization of a pH stable *Ganoderma fornicatum* laccase in *Pichia pastoris*

Wan-Ting Huang and Ching-Tsan Huang
Institute of Microbiology and Biochemistry, National Taiwan University
Taipei, Taiwan

P15 Optimization of the heterologous production of a *Rhizopus oryzae* lipase (ROL) in *Pichia pastoris* system using mixed substrates by controlled fed-batch bioprocess

Carolina Arnau, *Ramon Ramon, Carles Casas and Francisco Valero
Departament d'Enginyeria Química. ETSE. Universitat Autònoma de Barcelona
Bellaterra (Barcelona), Spain
** Bioingenium, S.L. Edifici Hèlix. Parc Científic de Barcelona*
Barcelona, Spain

P16 Secretion and proteolysis of heterologous proteins fused to the *E. coli* maltose binding protein in *Pichia pastoris*

Zhiguo Li[#], Wilson Leung*, Amy Yon*, Vincent C. Perez*, John Nguyen*, Jane Vu*, William Giang*, Linda Luong*, Kate A. Salazar*, Tracy Phan*, Seth R. Gomez*, Andreas Franz[#], Geoff P. Lin-Cereghino*, and Joan Lin-Cereghino*
** Department of Biological Sciences, University of the Pacific*
Stockton, California
 # Department of Chemistry, University of the Pacific
Stockton, California

P17 P*ichia* Glycoswitch™ technology: switching the N-glycosylation

Tiels P, Deschutter K, Van West E, Callewaert N
VIB
Ghent, Belgium

P18 Glyco-systems engineering of the non-conventional yeast /*Yarrowia lipolytica*/ for homogenous glycoprotein modification with the universal Man3GlcNAc2 N-glycan precursor.

Karen De Pourcq[1] , Annelies Van Hecke[1] and Nico Callewaert[1,2].
[1] *Department for Molecular Biomedical Research, VIB*
Ghent, Belgium.
[2] *Department of Biochemistry, Physiology and Microbiology, Ghent University*
Ghent, Belgium.

Abstracts – Oral Presentations

T1 *Pichia:* A historic perspective with a look to the future

James M. Cregg

Keck Graduate Institute of Applied Life Sciences
Claremont, CA

As systems for the production of recombinant proteins, yeasts combine the growth and genetic manipulation advantages of bacteria with the ability to perform important post-translational modifications. Relative to *Saccharomyces cerevisiae*, *Pichia pastoris* has two significant advantages as a host. The first is the promoter used to transcribe most foreign genes, which is derived from the *P. pastoris* alcohol oxidase I gene (*AOX1*). This promoter is efficiently transcribed in cells exposed to methanol as the sole carbon source but is highly repressed under most other growth conditions. The second advantage is that *P. pastoris* does not have a tendency to ferment as does *S. cerevisiae*. A product of fermentation is ethanol, which can rapidly build to toxic levels in high-density cultures. As a consequence of these and other advantages, *P. pastoris* expression strains are easy to scale up from shake-flask cultures to large-volume fermenter cultures growing at cell densities of greater than 100 grams/liter, dry cell weight. The *P. pastoris* system is particularly valued for its ability to secrete recombinant proteins. Since the organism secretes only low levels of native proteins, the recombinant protein is often the major protein species in the medium.

Equally important to the success of *P. pastoris* as an expression system is its ready availability from a commercial source (Invitrogen, Carlsbad, CA USA). Strains, expression vectors and instructions can be purchased at a nominal cost by all labs.

T2 Entering the genomic era: the *Pichia pastoris* genomic sequence and a look into the information within.

Kristof De Schutter[1,2], Petra Tiels[1,3], Yao-Cheng Lin[4,5], Annelies Van Hecke[1,3], Pierre Rouzé[4,5], Yves Van de Peer[4,5] and Nico Callewaert[1,3]

[1] *Unit for Molecular Glycobiology, Department for Molecular Biomedical Research, VIB, Ghent-Zwijnaarde, Belgium*
[2] *Department for Biomedical Molecular Biology, Ghent University, Ghent-Zwijnaarde, Belgium*
[3] *Unit for Molecular Glycobiology, L-ProBE, Department of Biochemistry and Microbiology, Ghent University, Ghent-Zwijnaarde, Belgium*
[4] *Department of Plant Systems Biology, VIB, Ghent-Zwijnaarde, Belgium*
[5] *Departmernt of Plant Biotechnology and Genetics, Ghent University, Ghent-Zwijnaarde, Belgium*

The methylothropic yeast *Pichia pastoris* is one of the most commonly used organisms for the production of recombinant proteins, its importance only increasing with the development of strains with fully humanised N-glycosylation. Despite its academic and industrial importance, relative few genetic tools or engineered strains have been generated. Here we remove a major stumbling block that has hampered studies and engineering of *Pichia pastoris* in providing its 9.43 Mbp genome sequence.
We identified many genes that are important for post-translational modifications, such as N- and O-glycosylation, the secretory pathway, and protein folding and degradation. These will be the target for the engineering of optimized protein production strains. Further, the analysis of the codon usage bias and endogenous signal sequences will be useful for the design of optimized constructs. The analysis of promoter regions will allow for the development of new promoters for the expression of heterologous proteins.
It is clear that the information obtained from the genome sequence will lead to the development of new tools and strains that will allow an increase in the productivity, homogeneity and stability of the *Pichia pastoris* expression system.

Reference:

De Schutter K, Lin YC, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouzé P, Van de Peer Y, Callewaert N. Genome sequence of the recombinant protein production host *Pichia pastoris*. **Nat Biotechnol**. 2009 Jun;27(6):561-6. Epub 2009 May 24.

Annotation website: http://bioinformatics.psb.ugent.be/webtools/bogas/

T3 New tools for accelerated protein expression and engineering using *Pichia pastoris*

Anton Glieder

Research Centre Applied Biocatalysis
Graz, Austria

Development times for improved enzymes and biopharmaceuticals (especially if derived from higher eukaryotes) by molecular engineering are too long. Therefore we need new tools which allow an accelerated discovery, supply and development of efficient and robust enzymes for industrial biotechnology, for the evaluation of the optimal expression construct and for the development of new or improved biopharmaceuticals. For example, directed evolution using the highly productive yeast *Pichia pastoris* directly as a host for libraries of protein variants, too, helps to avoid the detouring, error prone library amplification and screening in *E. coli.*

A few years ago we developed fast and simple methods to screen *Pichia pastoris* expression clones for improved enzyme activity and/or selectivity. This was a first step towards using *Pichia pastoris* as a host for directed evolution (of eukaryotic enzymes) and we were able to verify the scalability of these findings (1-3 m3). In order to avoid time consuming and inefficient steps in expression clone construction, such as subcloning of genes into expression plasmids, ligation and DNA amplification in *E. coli*, gene disruption in *Pichia pastoris* and co-expression of other target genes and genes for helper proteins we developed

- New host strains for efficient transformation and expression and for reliable screening of new enzyme variants, containing partially integrated selection markers and other properties which allow to speed-up library generation/screening cycles during enzyme development and homologous recombination.

- New, ligation independent methods for direct linear expression cassette construction and host cell transformation by PCR in combination with new screening hosts allowing efficient and fast strain construction for the expression and analysis of new enzyme variants (This is especially important for the work with systems like *Pichia pastoris*, which employ gene integration into the genome).

- A toolbox of natural, engineered and synthetic promoters, signal sequences, fusion partners, selection markers and helper proteins to identify the optimal expression strategy by combinatorial approaches and screening.

The applicability of these new vectors, strains, tools and strategies was demonstrated by several examples about expression strain construction and enzyme engineering.

T4 Identification of hypoxia-inducible promoters in *Pichia pastoris*

Andrea Camattari, Dave Siak-Wei Ow and Victor Vai-Tak Wong

Bioprocessing Technology Institute
Singapore

The typical expression system for *Pichia pastoris* is based on the methanol – inducible promoter of Alcohol Oxidase 1 gene (AOX1). Although such a promoter is generally efficient and able to sustain a high yield of product, the inducer for AOX1, methanol, appears problematic due to handling issues (especially in large scale productions). Moreover, the cost of methanol is destined to increase, being derived from oil processing.

To develop novel promoters and processes, we focused our attention to alternative means of inducing protein production. In particular, we tested the possibility of using hypoxia in bioreactor, representing a cheap and easy alternative to methanol addition, and induce hypoxia – regulated promoters.

Using a dual strategy, involving 2D gel separation and differential labeling (iTraq), several proteins, induced under low oxygen condition, have been identified; qPCR experiments proved the transcriptional nature of the induction.

The promoters from a subset of promising genes have been cloned upstream a reporter gene, and promoter activities have been evaluated in lab scale process; although some optimization is required, the potentiality for such an expression system is confirmed. The application of hypoxia promoters for the expression of industrially relevant proteins will be tested in the next step of development

T5 Going double-digit with *Pichia*: high-level expression of human serum albumin and transferrin as well as fusion proteins driven by AOX1 promoter library

Roland Weis

VTU Technology
Grambach, Austria

Although *Pichia pastoris* nowadays is a state-of-the-art host for recombinant expression with extraordinary capabilities for the secretion of heterologous proteins, only few examples of expression titers as high as >5g/L are reported.

We report recombinant protein yields exceeding 10 g/L employing our proprietary AOX1-promoter library for expression of human serum albumin, human serum transferring(s) as well as fusion proteins thereof. Different cloning and expression strategies were assessed to improve expression titers, and parameter scouting in 1L bioreactors resulted in an optimized fermentation protocol, executed in 5L fermentations. As a result of downstreaming approaches, high purity and quality of the described proteins was achieved.

Relying on the AOX1-promoter library, several other (human) recombinant proteins were expressed to titers between 2 and 7 g/L.

T6 *Pichia* fermentation: past, present and future

Dr. Michael Meagher

University of Nebraska
Lincoln, NE

This talk will provide a description of the evolution of *Pichia* fermentation development and scale-up over the last 20 years. The approach to running *Pichia* fermentation to optimize production of a recombinant protein has come along ways since the "Invitrogen Process," which is a DO stat method for an AOX1-based fermentation and actually predates Invitrogen. Since that time approaches based around methanol sensors and growth rate dependent models have been used successfully to optimized and scale-up methanol-based *Pichia* fermentation process. Other non-methanol promoter systems have also been used successfully, including the GAP promoter, which is more amenable to scale-up, eliminating the requirement for methanol. A review of the different fermentation strategies, the advantages and disadvantages of these strategies and the future for Pichia fermentation will be presented.

T7 *Pichia* technology at the cutting edge: process development meets strain design

Karin Kovar

Institute of Biotechnology
Zurich University of Applied Sciences (ZHAW)
Switzerland

When developing recombinant products, strain construction is often started before closer considerations of the cultivation step are made. Thus, any problems arising during process development in a bioreactor are frequently solved by technological (bioprocessing) means rather than by revising the strategy for strain design and host selection. The following fields of research in *Pichia* provide examples of beneficial interaction between process technology and molecular biology:

- elucidation of new regulation mechanisms for synthetic promoters in methanol-free but inducible processes;
- establishing a link between the physiological state of cells and the quality of the expressed heterologous protein, achieving proper protein conformation and/or homogenous glycosylation structures in glyco-engineered strains while avoiding degradation products;
- using physiological analyses at single cell level and thereby radically changing the paradigm in the commonly used practice of bioprocess monitoring, which fails to inform on the state of individual cells within apparently uniform cultures in a bioreactor.

The resulting technology-driven concepts (multiple substrates/elements limitation, quantification of the physiological state, evaluation of large data quantities, etc.) have been developed and qualified with relevant reporter molecules such as industrial enzymes, therapeutic proteins and proteinaceous biomaterials. The large data source now available suggests several generic approaches in process development applicable to particular product families without the need for *de novo* development on a protein-by-protein basis.

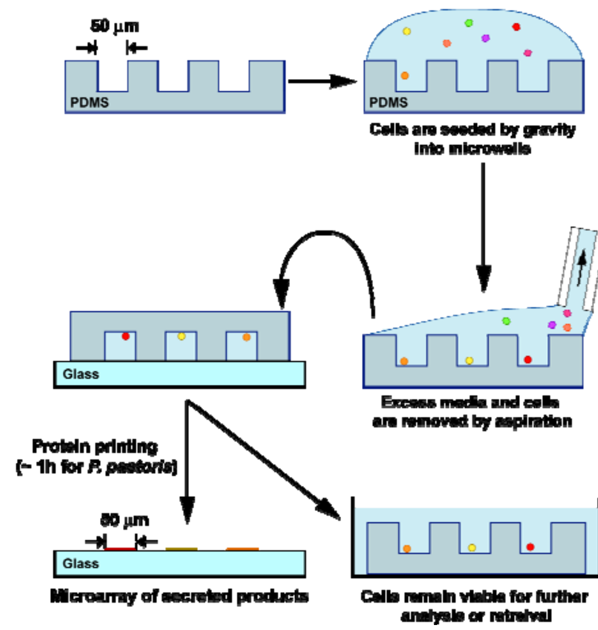
T8 Single-cell analysis of protein expression and secretion in *Pichia pastoris* by microengraving

Kerry Routenberg Love[1], Bo Jiang[2], Terrance Stadheim[2], and J. Christopher Love[1]

[1]*Dept. of Chemical Engineering, Massachusetts Institute of Technology*
Cambridge, MA
[2]*GlycoFi, Inc.*
Lebanon, NH

Maximizing the production of proteins either by mammalian cell culture or by microbial fermentation requires periodic assessments to analyze both the quantity and quality of the material produced. Iterations of this assessment in response to changes in growth media, culture density or other variables can improve fermentation conditions, and thus, increase protein expression. Much time is therefore spent in industry optimizing fermentation conditions for a given clonal line, but without attention to how heterogeneity in production amongst the cells themselves affects the overall yield of protein. Examining a clonal line at the single cell level makes it possible to study the distribution of protein production under a given set of conditions and may identify certain variants within the population that have desirable properties. One such property is the ability of the expression host to secrete robust amounts of protein during cultivation. We have adapted the microengraving technique, which has previously been used to screen large libraries of hybridomas for clones secreting specific antibodies of interest, to assess protein secretion from *P. pastoris*.

Yeast cells are deposited into a polymeric biochip containing a dense array of 50 μm wells and used to "print" secreted proteins onto a glass slide (see figure). The resulting protein array can be correlated with the cells in the device. These data enable the identification and subsequent retrieval of clones based on their ability to secrete a particular protein product. We have examined the utility of this technique using *P. pastoris* to understand a variety of questions, including: What percent of a population is secreting at the single cell level under a given set of batch growth conditions? Does the percentage of secreting cells change using different promoter systems? How does protein secretion track with cell growth? Can secretory capabilities be inherited within an otherwise clonal population of cells, or are superior clones only achieved using environmental pressure (mutation or selection)? These results will be presented.


50 μm
PDMS
PDMS
Cells are seeded by gravity into microwells
Glass
Excess media and cells are removed by aspiration
Protein printing (~ 1h for P. pastoris)
50 μm
Glass
Microarray of secreted products
Cells remain viable for further analysis or retreival

T9 A scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime

Richard A.J. Darby, William Holmes & Roslyn M. Bill

Aston University, UK

Optimizing the scale-up of process conditions to result in high yields of recombinant proteins is an enduring bottleneck in contemporary bioscience. Typically researchers rely on varying selected parameters through repeated rounds of trial-and-error. We have adopted a rational 'design of experiments' (DoE) approach to generate a predictive model that describes small-scale screens that is scalable to bioreactors.

We present a DoE approach using a multi-well mini-bioreactor to establish high yielding production phase conditions that predictably scale to a 7 L bioreactor. Using secreted GFP we derived a model of protein yield as a function of temperature, pH and percentage dissolved oxygen in the culture medium. Importantly, when the yield was normalized to culture volume and density, the model was scalable from mL to L working volumes. Increasing the pre-induction biomass improved the model-predicted yields further and yields were most improved by varying the fed-batch induction regime to minimize methanol accumulation, so that the productivity of the culture increased throughout the whole induction period.

Using a DoE approach reduces overall process development time.

T10 On the way to a self optimizing fully automated and global observable integrated bioplant for production of recombinant proteins with *Pichia pastoris*

R. Luttmann[1], K. Loegering[1], J. Fricke[1], S. Martens[1], C.Mueller[1], S.-O. Borchert[1], H.-P. Bertelsen[1], U. Scheffler[1], B. Faber[2], G. Cornelissen[1]

1 Research Center of Bioprocess Engineering and Analytical Techniques HAW – Hamburg University of Applied Sciences
Hamburg, Germany
2 Biomedical Primate Research Center, Department of Parasitology
Rijswijk, The Netherlands

The dream of every biochemical engineer is a fully automated plant which combines up-stream and down-stream unit operations to an Integrated Bioprocess. Moreover measuring possibilities of all relevant cell external process variables and parameter as well as cell internal physiological states should allow a global process observation and enable online procedures for process optimization.

This contribution describes several steps to this ambitious goal, demonstrated by examples from production of Lipase B from *Candida antarctica* (CALB), a truncated human Chemokine Interferon-inducible T-cell -chemoattractant (1-3del I-TAC) and a possible Malaria vaccine candidate Apical Membrane Antigen 1 from *Plasmodium falciparum* (PfAMA1).

Starting with the development of fully automated multi-stage production with standard bioreactors where PAT applications are introduced in research and production reactors (high instrumentation). Based on recombinant protein and glycerol detection via atline process-HPLC's and on cell density plus cell viability inline measurements volumetric and cell specific reaction rates were calculated and observed quasi online.

The study of resulting expression dynamics leads to a proper mathematical description and to concepts of Integrated Bioprocessing. This yields in industrial developments of appropriate equipment with Sartorius Stedim Systems and GEA Westfalia Separator.
First a new reactor concept was realized for a repeated fed batch protein production in the bioreactor and integrated simultaneous protein separation steps via cross flow filtration, which are operated sequentially.

Another example is the development of a fully automated production strategy in a scale-down production plant for repeated high cell density cultivations and sequentially integrated cell separations with a new developed separator SC1 and additional protein purification procedures with an ÄKTApurifier 100 chromatography system. At both plants the recombinant protein was measured at three different places.

Applications of Design of Experiment (DoE) enable post experimental identification of optimal expression parameters. Screening and optimization runs are carried out in one bioreactor (sequential DoE) as well as in a 6 fold multifermenter system with a seventh mother fermenter for fresh cell production (sequential/parallel DoE).

Moreover atline measurements of product concentration and/or product quality and a modern automation structure opens a wide field of online optimization in the recombinant protein production plants by application of genetic algorithm or the simplex strategy from Nelder/Mead.

T11 Protein N-glycan engineering in *Pichia pastoris* for increased homogeneity and mammalian use: an introduction and early results

Roland Contreras

Ghent University and Flanders Interuniversity Institute for Biotechnology
Ghent, Belgium.

Protein linked N-glycans have a wide range of functions and changes in their structure has been linked several times to human disease and several other complex biological phenomena like development and ageing . Especially remarkable is their complexity and their heterogeneity, two aspects that are poorly understood. The relationship between complexity, heterogeneity, and functional diversity or pleiothrophy has not been studied well. To answer complexity and heterogeneity questions, we have developed systems to produce homogeneous, well defined engineered protein N-glycans.

Lower eukaryotes have been used at early moments in our group for glycan engineering as they only synthesize N-glycans of the high-mannose type. Thus, they are excellent hosts for this engineering work and they don't have competing complex glycosyl tranferases coded for in their genome as is the case in widely used mammalian cell lines (e.g. CHO). Redirection of the fungal pathway, even to a simple hybrid or complex mammalian type structure requires several genetic interventions such as gene knock-outs and heterologous expression of mammalian glycosyl transferases. Furthermore, additional in vitro enzymatic manipulations may be required.
Several human and rodent glycoproteins have been expressed with excellent efficiency in our glycol-engineered strains.

Further, second/third stage strain improvements will be discussed by Dr. Callewaert.

T12 Engineering complex-type N-glycosylation in *Pichia pastoris* using *Pichia* GlycoSwitch^{TM} technology

Pieter Jacobs, Kristof De Schutter, Roland Contreras, Nico Callewaert

Department for Molecular Biomedical Research, VIB
Department for Biomedical Molecular Biology, UGent
Department of Biochemistry and Microbiology, UGent
Ghent-Zwijnaarde, Belgium.

The range of applications for recombinant glycoprotein production in yeasts is limited by heterogenous N-glycosylation of the high-mannose type. These highly heterogenous glycans severely reduce the efficiency of downstream processing, often necessitate enzymatic removal before crystallography, and can be detrimental to the therapeutic efficacy of biopharmaceuticals.
To alleviate this problem, we have engineered the human biosynthetic pathway for biantennary galactosylated glycans into *Pichia pastoris* over the past several years. Along the way, engineered strains were also obtained which homogenously modify their glycoproteins with the human intermediates Man_5Gn_2 and the hybrid-type $GalGnMan_5Gn_2$, which are useful in themselves for many purposes. The engineering goal of >85% conversion efficiency at each step of the pathway was achieved, resulting in a final glycosylation profile which looks very similar to the one present on human serum IgG, except for the absence of core-fucosylation. This latter aspect is a desired feature in the production of mAbs which operate therapeutically through ADCC.
We will present data on GlycoSwitch *Pichia* expression of several human and murine cytokines, spanning about 2 orders of magnitude in expression level, from low mg/l to gram/l, demonstrating that the introduced pathway can efficiently handle large glycoprotein fluxes. We will also present initial data on high-cell density cultivation of the Man_5Gn_2 strain.

T13 Expression of variable and invariable trypanosomal glycoproteins in *Pichia pastoris* for diagnosis of human African trypanosomiasis and surra

[1]Stijn Rogé[1], Wouter Vervecken[2,3,4], Yves Guisez[5] and Philippe Büscher[1]

[1] *Unit of Parasite Diagnostics, Department of Parasitology, Institute of Tropical Medicine Antwerp, Belgium*
[2] *Department for Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent-Zwijnaarde, Belgium.*
[3] *Department of Biomedical Molecular Biology, Ghent University, Ghent-Zwijnaarde, Belgium.*
[4] *Currently working at Oxyrane Belgium nv Ghent-Zwijnaarde, Belgium.*
[5] *Laboratory for Molecular Plant Physiology and Biotechnology, Department of Biology, University of Antwerp Antwerp, Belgium.*

Human African trypanosomiasis (sleeping sickness) is caused by *Trypanosoma brucei gambiense* (chronic) or *T.b. rhodesiense* (acute) and occurs only in Sub-Saharan Africa where its vector, the tsetse fly occurs. Surra is a disease of domestic animals like horses, camels, bovines caused by *Trypanosoma evansi* and mechanically transmitted by blood sucking flies.

Variable surface glycoproteins (VSGs) of *Trypanosoma brucei gambiense* (LiTat 1.3) and *Trypanosoma evansi* (RoTat 1.2) and the 75kDa and 65kDa invariable trypanosome surface glycoproteins (ISG 75 and ISG 65) were amplified from cDNA, cloned in pPIC9, sequenced and electroporated in the yeast *Pichia pastoris* (Mannose 5 strain). The cells were grown in BMGY medium at 29°C and the expression was induced in BMMY medium (with 2% casamino acids) at 15°C. The recombinant proteins contain a C-terminal His tag (VSGs) or E tag (ISGs) for column purification and are secreted in the supernatant at 2 to 10 mg per litre cell culture.

In ELISA with sera from experimentally infected goats, the purified recombinant RoTat 1.2 and ISG 75 show reactivities that are comparable with the native purified RoTat 1.2, thus presenting an alternative to antigen production by mass culture of *T. evansi* in laboratory rodents. The purified recombinant proteins are currently tested in ELISA with human and animal sera to assess their diagnostic potential for human sleeping sickness and surra.

This study received financial support from Flanders Institute for Research and Technology, Flanders Fund for Scientific Research, Directorate General for Development Cooperation and the Secondary Research Funding of the Institute of Tropical Medicine (SOFI-B).

T14 Difficult proteins: expression and secretion in *Pichia pastoris* using novel sumo system

Raymond J. Peroutka, Yilin Yan, Lauren E. Reuther, James E. Strickler
and Tauseef R. Butt,

LifeSensors, Inc.
Malvern, PA

The methylotrophic yeast *Pichia pastoris* has gained popularity as an expression host for its ability to produce, in many cases, very large amounts of recombinant protein. Production of recombinant protein in P. pastoris is however highly variable and greatly influenced by the inherent properties of the product of interest. To overcome these difficulties we have engineered several small ubiquitin-like modifying (SUMO) proteins for use as fusion partners. The covalent attachment of SUMO, via an isopeptide bond, to a variety of proteins *in vivo* is a eukaryotic posttranslational event demonstrated to play a critical regulatory role; thus, the machinery for attachment and deconjugation of SUMO is found in all eukaryotic cells. Mutants have been designed to overcome endogenous deconjugation while enhancing protein production and secretion. Additionally, we have engineered new desumoylases that cleave the mutant SUMO-fusions to efficiently generate native proteins. We will present examples of several proteins produced as SUMO fusions were the amount of secreted product was increased 4 to 47 fold. Development of a novel SUMO system for P. pastoris offers the advantages of expressing toxic or difficult-to-express proteins in quantities that were previously impossible.

T15 Expression of membrane-bound monoamine oxidases from three different organisms in *Pichia pastoris*

Jin Wang, Milagros Aldeco , Dale E. Edmondson

Department of Biochemistry, Emory University School of Medicine
Atlanta, GA

Monoamine Oxidases A and B are flavin-containing mitochondrial membrane-bound enzymes which catalyze the oxidative degradation of biogenic and xenobiotic amines and react with O_2 to form H_2O_2. Due to their involvement in age-related neurodegenerative diseases (e.g. Parkinson Disease and Alzheimer's Disease), studies requiring large quantities of purified enzymes are required for development of inhibitors that could be developed as neuro-protectants.

To date, we have successfully expressed Human MAO A and B, Rat MAO A and B and Zebra fish (*Danio rerio*) MAO, as well as a number of mutants in *Pichia pastoris*. The genes encoding these enzymes are expressed under the control of the methanol oxidase promoter. These enzymes are all found to be localized in the outer mitochondrial membrane of *Pichia*. One liter fermentation cultures yield 220 grams (wet weight) of cells from which ~200 milligrams of functional protein is purified. The level of MAO situated in the mitochondria approached 50% of the total protein in this organelle. These results demonstrate the utility of the *Pichia* system for the expression of large quantities of membrane associated enzymes from humans to teleosts.

This work was supported by NIH grant GM-29433.

T16 Transcriptomics-based engineering of *Pichia pastoris* for improved production of eukaryotic membrane proteins

Maria Freigassner[1], Gerhard Thallinger[2], David Drew[3], Harald Pichler[1], James Cregg[4], Anton Glieder[1]

[1] *Institute for Molecular Biotechnology, Graz University of Technology*
Graz, Austria
[2] *Institute for Genomics and Bioinformatics, Graz University of Technology*
Graz, Austria
[3] *Membrane Protein Crystallography Group, Division of Molecular Biosciences, Imperial College London, UK*
[4] *Keck Graduate Institute, Claremont Colleges*
Claremont, CA

The methylotrophic yeast *Pichia pastoris* ranks among the most successful expression systems for eukaryotic membrane protein production, as judged from the number of currently available structures. Though it is a well-established system capable of providing mg amounts of properly folded, active and crystal-grade membrane proteins, information on physiological responses upon overexpression of eukaryotic membrane proteins is still scarce.
In order to shed some light on this, we analyzed *Pichia pastoris'* transcript pattern during expression of several model proteins, all being eukaryotic membrane proteins, by employing newly designed Affymetrix microarrays. mRNA profiling provided evidence that different membrane proteins evoked different responses on the molecular level, depending on localization and hence pathways used during translation and trafficking.
Based on these data we identified proteins which play a crucial role during membrane protein biosynthesis and rationally analyzed co-expression effects on the biogenesis of eukaryotic membrane proteins. One key to success for increased membrane protein production efficiency lies in an improved cellular capacity to proliferate extra internal membranes.

T17 Expanding the genetic repertoire of *Pichia pastoris*

Travis S. Young, and Peter G. Schultz

Department of Chemistry, The Scripps Research Institute
La Jolla, CA

The addition of novel amino acids, beyond the canonical 20, to the genetic codes of *E. coli*, *S. cerevisiae,* and mammalian cells has allowed the expression of recombinant proteins with exquisite control over chemical structure. Whereas these systems have been highly successful at the *in vivo* expression of a wide variety of proteins containing unique amino acids, there remain proteins whose expressions are not feasible in the existing systems. To allow access of the unnatural amino acid methodology to these proteins as well as increase the overall yields mutagenized proteins, we have developed a system to genetically encode the unnatural amino acid machinery in *Pichia pastoris*. This system has successfully added 8 unnatural amino acids containing fluorophores, photoactive, heavy metal, structural probes, or uniquely reactive groups to the genetic repertoire of *P. pastoris*. Of particular interest, the unique ketone functionality of *p*-acetylphenylalanine (*p*AcF) undergoes highly selective, orthogonal ligations under mild conditions. We have incorporated *p*AcF into human serum albumin expressed in *P. pastoris* and used it to covalently attach therapeutic peptides which suffer from short *in vivo* serum half lives. In addition this technology is currently being applied to full length antibodies and other glycosylated proteins.

T18 Bench to bedside with *Pichia:* recombinant antibody-based cancer treatments

Kerry Chester

University College
London, UK

The GMP production Facilty at UCL uses the *Pichia* expression system for production of clinical grade antibody-based therapeutics. To achive this we have developed procedures that interface *Pichia pastoris* fermentation and soluble expression with capture of secreted hexa-histidine-tagged proteins directly from fermentation broth. The production procedure has been successful in generating a complex, dimeric, single chain Fv (scFv) antibody-enzyme fusion protein for use in antibody directed enzyme prodrug therapy (ADEPT) of cancer. The recombinant product has post translational N-mannosylation to mediate rapid clearance from non-cancerous tissue. The purified product was stable, free from detectable contaminants, biologically active, safe and well tolerated in man where it has shown preliminary evidence of efficacy in Phase I/II ADEPT trials. The pharmacokinetics in patients are comparable to those obtained in murine models and support experimental evidence of clearance via the mannose receptor.

The GMP expression/purification procedure has also been successfully applied to generate scFvs in monomeric and diabody format. Our results indicate that the *Pichia* system provides an excellent platform for production of biotherapeutics for imaging and therapy of cancer.

T19 Discovery and development of Ecallantide

Mark de Souza

Dyax Corp., MA

Dyax discovered Ecallantide, a potent inhibitor of human plasma kallikrein (Ki = 25 pM), from a phage display library based on a natural human protease inhibitor scaffold, the first Kunitz domain of tissue factory pathway inhibitor. Ecallantide has been evaluated as a subcutaneous injection to treat acute attacks of hereditary angioedema (HAE) in two Phase 3 placebo-controlled trials. Dyax has submitted a BLA for ecallantide in HAE and the FDA has assigned Dyax a PDUFA date of December 1, 2009. Ecallantide is the first therapeutic protein expressed in *Pichia pastoris* that has advanced through clinical trials to BLA filing in the US and offers significant potential to treat hereditary angioedema and other indications with unmet medical need.

T20 Manufacturing of proteins in *Pichia* - present and future

Gesche Bernhard, Sandra Nunez, Joachim Klein, Jana Wenger, Torsten Schmidt, Diego Schmidhalter,

Lonza Ltd.
Visp, Switzerland

With features like secretion of soluble functional products, simple nutritional requirements, growth to high cell densities, posttranslational modifications including glycosylation yeasts like the methylotrophic *Pichia pastoris* are attractive expression systems for the production of recombinant proteins and peptides.

As a custom manufacturing organization, Lonza uses optimised high-cell-density *Pichia pastoris* processes for the production of recombinant proteins from lab scale to production scale. Oxygen consumption, heat evolution and by-product formation are important issues in bioprocess scale-up and industrial manufacturing.

At Lonza, several catalytic and also therapeutic recombinant proteins have been produced by *Pichia* under the control of the strong and tightly regulated alcohol oxidase promoter (AOX1) and under Lonza`s proprietary, constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (GAP), respectively.

Using *Pichia* based expression systems cost of goods (CODS) could be reduced as product is released into the fermentation medium and downstream processing can be therefore considerably simplified. To further maximize productivity in high-cell-density fermentations both fermentation procedures and strain construction strategies including high-throughput clone selection has to be optimized. One of the most promising and trendsetting developments is, however, that the N-glycosylation machinery of *Pichia* can be engineered to produce proteins with defined complex and homogenous glycosylation pattern.

T21 *Pichia pastoris* whole cell biocatalyst

Abad, S.[1], Nahalka J.[1], Schroer K.[1], Hartner, F.S.[1], Pscheidt B.[1], Luef K.[1], Nidetzky B.[2] and Glieder A.[1,3]

[1] *Research Centre Applied Biocatalysis, Graz, Austria*
[2] *Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria*
[3] *Institute of Molecular Biotechnology, Graz University of Technology Graz, Austria*

Efficient recombinant protein expression in *Pichia pastoris* is well documented. The development of a simple molecular biology toolbox for this yeast including the strong and inducible AOX1 promoter, the α-mating factor secretion signal, high trough put cultivations, well-established high cell density cultivation as well as the availability of the genome sequence makes this yeast a perfect industrial expression host. Nevertheless, in other fields such as whole cell biotransformation *Saccharomyces cerevisiae* is still dominating.

Our focus is to employ and adapt *Pichia pastoris* into an attractive whole cell factory. To achieve this we pursued from two different starting points the efficient performance of *Pichia pastoris*. On the one hand, we used the presence of endogenous catalase and employed permeabilized recombinant *Pichia pastoris* cells as shelter for oxygen interface sensitive oxidases. The expression level of the target enzyme was improved by changing the codon usage and optimization of the peroxisomal targeting signal (PTS1). Therefore the methanol utilization pathway provides two advantages during MeOH induction. The regulation of the AOX1 promoter for high level recombinant expression as well as the proliferation of peroxisomes offering enough space, shelter and the right oxidative environment for our target enzyme, including catalase for H_2O_2 detoxification and O_2 regeneration.

On the other hand the already mentioned MeOH dissimilation pathway in *Pichia pastoris* has been engineered towards cofactor regeneration for reductive biotransformation. In most cases the requirement of stoichiometric amounts of reduced cofactor nicotinamide adenine dinucleotide (NADH or NADPH) for oxidoreductases is the main drawback for industrial processes. Therefore cofactor recycling becomes a major topic for the production of important building blocks for fine chemicals. Our approach, the deletion of the two dihydoxyacetone synthase genes redirects the metabolic flux of *Pichia pastoris* towards an industrialized minimal metabolism. In this way highly efficient NADH supply is boosted when MeOH is added as sole carbon source, while catalyst expression is induced at the same time and conversion of the carbon source to biomass is minimized. In parallel, the major bottleneck (identified by kinetic modelling) of the MeOH utilization pathway was removed by overexpression of *P.pastoris* formaldehyde dehydrogenase (FLD), thus increasing the efficiency of alcohol dehydrogenase based bioreductions.

These examples provide two show cases for possible industrial applications of *Pichia pastoris* in new fields.

T22 The Glycoprotein hormones, receptors and *Pichia pastoris*

Rajan R. Dighe

Indian Institute of Science
Bangalore, India

The glycoprotein hormones and their receptors are interesting models for studying the protein-protein interactions, signaling mechanisms and structure-function relationship of complex glycoproteins. The hormones are heterodimeric proteins with an identical α subunit associated noncovalently with the hormone specific β subunits. Both subunits have several disulfide bridges, cystine knots and the c terminus of β subunit wrapping around the α subunit forming a seat belt like structure. Both subunits are glycosylated with core glycosylation being absolutely essential for the correct folding of each subunit. The receptors for these hormones belong to the family of GPCR with a large, glycosylated extracellular domain responsible for hormone binding and a characteristic 7 transmembrane domain involved in signal transduction. The major clinical applications of these hormones are treatment of infertility and thyroid disorders and immunocontraception.

Over last several years, the laboratory has successfully expressed all the four human glycoprotein hormones using the Pichia expression system employing both AOX1 and GAP promoters. The hormones expressed were shown to be properly folded as judged by monoclonal antibody based RIAs, receptorassays and in vitro bioassays. Employing new robust fermentation protocols developed in the laboratory, yields of the recombinant hormones were increased to approximately 40-45 mg/liter. A fermentation protocol for producing the ^{15}N labeled hormone molecules using ^{15}N ammonium sulfate as the nitrogen source has also been developed. Reproducible purification methodologies have allowed to us purify large quantities of highly purified recombinant hCG and hFSH. Determination of the structure of the glycan moiety revealed presence of 9 to 12 mannose residues with GS115 while more than 20 mannose residues were present when X33 was used as the host strain. The glycan moiety also showed presence of phosphorylated sugar residues. While the hormones produced by both strains were biologically active *in vitro*, their *in vivo* bioactivities were severely compromised as the proteins were cleared very fast from the circulation. Thus, these hormones are very interesting models for glycoengineering by the humanized Pichia strains.

The laboratory has also expressed and purified agonist and antagonists of hCG and hFSH that have provided interesting insights into the mechanism of hormone-receptor interactions. The hCG antagonist (hCGββ) has a potential for treatment of a rare genetic disease of precocious puberty. In addition, the laboratory has expressed and purified extracellular domains of all three human glycoprotein hormone receptors that are being used to study the hormone-receptor interactions and generate interesting polyclonal, monoclonal and recombinant antibodies with interesting clinical applications.

Interestingly, the Pichia system was also used to produce and purify human Growth hormone with yields of 500 mg/liter.

(Supported by grants from the DBT, DST and UGC, Government of India, New Delhi).

T23 Characterisation and scale up of *Pichia pastoris* processes for biopharmaceutical production

IJ Hodgson, BV Kara, JM Liddell, T Williamson

Avecia Biologics
Billingham, UK

While the *Pichia pastoris* expression system has been in development for almost 35 years, there have been few reports of the characterisation and scale up of *Pichia pastoris* processes to produce biopharmaceuticals. With extensive experience of development and scale up of such processes, gained over many years, Avecia Biologics is well placed to discuss appropriate approaches. With the first biotherapeutics produced using *Pichia pastoris* close to achieving regulatory approval it is timely to review approaches to development, characterisation and scale up of *Pichia pastoris* fermentations as well as how high biomass fermentation processes can interface with purification processes.
Approaches to identify important parameters controlling *Pichia pastoris* fermentation performance will be discussed and successful implementation in scaling and validating fermentation processes at scales up to multi-thousand liter scale.

As a consequence of the high biomass of *Pichia pastoris* fermentations purification presents particular challenges. Two different approaches to separation, the first a standard approach involving solid liquid separation/ ultrafiltration and packed bed capture and an alternative involving direct capture from fermentation culture (expanded bed chromatography) will be reviewed from the perspective of large scale data. As a result of experience gained, factors which might favour one primary separation route over another can be identified from some basic considerations of the target protein.

T24 Development of an automated cultivation process for the expression of recombinant house dust mite allergen Der p 1 in *Pichia pastoris*

G. Cornelissen, B. Weber, R. Luttmann, O. Cromwell, H. Fiebig, A.Nandy

University of Applied Sciences Hamburg
Hamburg, Germany

House dust mite allergens are one of the main causes of indoor allergen induced allergic diseases. Recombinant allergens have the potential to improve extract based specific immunotherapy regimen in future.

Dermatophagoides pteronyssinus Der p 1 is a major allergen associated with IgE-antibody frequencies of 70-80 % and, together with Der p 2, is the first choice for inclusion in a therapeutic cocktail of recombinant allergens. Here we describe the automation and optimization of the production process of proDer p 1 in P. pastoris.

Extensive on-line analysis was used to build up a fully automated cultivation process. The form of Der p 1 in the culture supernatant is effected by the choice of pH. The protein yield could be increased tenfold by fermentation at pH 6 and expression of proDer p 1 instead of mature Der p 1. Further increase could be reached by lowering the temperature during expression. An optimized process attains more than 1 g/L proDer p 1 in the media phase.

The concentration of proDer p 1 in the culture supernatant has been increased more than 20-fold. Process automation is an effective tool for optimization of the expression yield, and an important first step to build up a cost-effective industrial production.

T25 Production of wasp venom allergens in *Pichia pastoris*

Irina Borodina[1], Lars K. Poulsen[2], Jørgen Nedergaard Larsen[3], Ib Søndergaard[1]

[1]*Center for Microbial Biotechnology, Technical University of Denmark*
Kgs Lyngby, Denmark
[2]*Laboratory for Medical Allergology*
Copenhagen, Denmark
[3]*Research Department, ALK-Abelló A/S*
Hørsholm, Denmark

Allergy to wasp venom is the most common insect allergy in the temperate European region, including Denmark. Insect venom allergy can be treated by multiple subcutaneous injections with wasp venom. Venom is collected from living wasps by a tedious manual procedure. The venom vaccine is not very efficient for all of patients for unknown reasons; diagnosis of venom allergy is a problem as well due to cross-reactivities of non-specific IgE antibodies. Recombinant wasp venom allergens may provide more simple, reproducible, cheaper and possibly patient-fitted vaccines and improve allergy diagnosis. We used methylotrophic yeast *Pichia pastoris* as a host for production of the major yellow jacket venom allergens: phospholipase A1, hyaluronidase and antigen 5. The characterisation of the allergens and their suitability for therapeutical use will be presented.

T26 Evaluation of methylotrophic yeasts as efficient expression hosts for the production of a glycoprotein for vaccine application: *pastoris* or *angusta*: what's the difference?

Geert Deschamps

Innogenetics Biologicals, Solvay Pharmaceuticals
Belgium

The hepatitis C virus E1 envelope glycoprotein (HCV E1) was studied as a relevant candidate for a HCV vaccine. The E1 glycoprotein was expressed in and purified from different recombinant protein expression systems. A comparison of the protein expressed in the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (*Pichia pastoris*) was performed. In particular the glycosylation was studied in comparison to E1 glycoprotein expressed in a mammalian expression system and in recombinant *Saccharomyces cerevisiae*. Related to the presence of terminal α1-3 mannose residues in the high mannose type glycosylation substantial differences were detected between the different yeast expression systems. An even more pronounced differentiation could be made on presence or absence of hyperglycosylated protein.
The yeast expression system with the optimal features was selected for further process optimization. The glycoprotein expression was enhanced by optimization of the leader sequence. Outstanding performance was obtained with the chicken lysozyme leader sequence. The highly hydrophobic protein was efficiently targeted to the endoplasmic reticulum, correctly processed and glycosylated. The resultant glycoprotein was purified and reconstituted into protein particles. The protein particle characterization by biochemical, biophysical and immunological methods will be discussed.
Finally insight will be given in the performed process optimization studies leading to a high productive process suitable for the production clinical grade material for phase III studies and beyond.

Abstracts – Poster Presentations

P1 Characterization of house dust mite allergen Der p 1 expressed in *Pichia pastoris*

A Nandy, G. Cornelissen, M. Wald, H. Kahlert, O. Cromwell, H. Fiebig

Allergopharma Joachim Ganzer KG,
Reinbek, Germany

House dust mites are the major source of indoor allergens that can cause allergic rhinitis and asthma. D. pteronyssinus Der p 1 is a major mite allergen with a sensitisation frequency of 70-80% among mite allergic patients. Der p 1 is first produced as an inactive pre-pro enzyme and subsequently processed in an autocatalytic process to form a mature cysteine protease. Pro-forms of wild-type Der p 1, a glycosylation deficient mutant S54G, and an active site mutant C34A/S54G, have been fused to the Saccharomyces cerevisiae -factor signal sequence and expressed in the methylotrophic yeast P. pastoris. By variation of the culture conditions the proDer p 1 S54G transformed P. pastoris cells can be induced to produce either a 35 kDa proDer p 1 or the mature 25 kDa mDer p 1.

Alternatively, mDer p 1 has been obtained from purified proDer p 1 by a quantitative in vitro maturation process. The purified recombinant molecules were characterized by immunological and biochemical methods and compared with purified natural Der p 1. Recombinant non-glycosylated mDer p

1 and inactive proDer p 1 expressed in P. pastoris are promising candidates for allergen specific immunotherapy and for allergy diagnosis.

P2 Continuous, on-line monitoring of glycerol and methanol during P*ichia pastoris* fermentations using near infrared spectroscopy

Elizabeth R. Gibson[1], Jonathon T. Olesberg[1,2], Mark A. Arnold[1,2]

[1]*ASL Analytical, Inc, Coralville, IA*
[2]*Optical Science and Technology Center and Department of Chemistry, University of Iowa, Iowa City, IA*

Pichia pastoris is a highly successful system for producing a wide variety of heterologous proteins. The ability to closely monitor parameters affecting fermentation and protein expression will enhance the development of the *Pichia* platform. Key analytes are currently monitored using off-line analyses that are time consuming and labor intensive, which makes optimization of reactor productivity difficult. In this work, a continuous, on-line near infrared (NIR) monitor was used to track both glycerol and methanol concentrations in real-time during *Pichia pastoris* fermentations. The *Pichia* growth medium and cells were sampled continually from the bioreactor and passed through the optical cell of a solid-state spectrometer, where spectra were collected over the 2.0-2.5 μm wavelength range. Models for glycerol and methanol concentrations were developed using a net analyte signal calibration method. These models have been used for *forward prediction* of analyte concentrations and have been successfully applied to ten fermentations taking place over the course of one year. Additionally, changes in cell density or biomass within the reactor can be tracked using the NIR spectra. The NIR monitor has been used to provide closed-loop feedback control of both glycerol and methanol concentrations during fermentations.

P3 Bioprocess scale-up and optimisation of erythropoietin production in *Pichia pastoris*

Nagamani Bora and Roslyn Bill.

Aston University
Birmingham, UK.

The production of recombinant therapeutic glycoproteins is an active area of research and drug development. *Pichia pastoris* is the chosen expression host for a wide range of glycoproteins for its scalability, robust expression and its ability to perform post-translational modifications. Recombinant human erythropoietin was produced under the control of the AOX1 promoter in *Pichia pastoris* harbouring the pPICZα vector. Strategies to increase secretion levels like the generation of clones with progressively increasing copy number and expression screening in microtitre plates were implemented. Feasibility studies at small scale conducted in wild type X33 and in protease deficient SMD1168 strains showed better productivity at low aeration. Soluble expression of erythropoietin on scale up was further optimised by varying process parameters such as dissolved oxygen, pH, temperature and the feeding regime. Improved productivity and decreased degradation of the final product were observed by adopting post-induction, a mixed feed strategy using methanol with sorbitol in a protease deficient strain.

P4 Antifoams in fermentation processes

S J Routledge and R M Bill

Aston University
Birmingham, UK.

Foaming during fermentation reduces the efficiency of the process leading to increased costs and reduced productivity. Foaming can be overcome by use of chemical antifoaming agents, however their influence upon the growth of organisms and protein yield is poorly understood. The objective of this work is to evaluate the effects of different antifoams on green fluorescent protein production (GFP). Antifoam A, Antifoam C, J673A, P2000 and SB2121 were tested at different concentrations upon the growth characteristics of *Pichia pastoris* X33 strain producing GFP and the yield of protein in shake flasks over 48 hours. All antifoams tested increased the volumetric yield of GFP compared to controls. The most significant increases in specific yields calculated by normalizing to culture density were observed with antifoams A, C and J673A. These findings in small scale suggest that antifoams of different compositions such as silicone polymers and alcoxylated fatty acid esters may influence growth characteristics of host organisms, indirectly affecting the protein yield. Future research will investigate the mechanisms behind these findings which may alleviate the foaming problems encountered in scale up and establish better production conditions of recombinant protein production.

P5 Prolipase signal sequence stabilizes the expression of bovine trypsinogen in *Pichia pastoris*

Nagaraj Govindappa, Nandini Nataraj, Reena Darshan, Sanjay Tiwari, Partha Hazra, Mukesh Patale, Pritish Sarkar, Gokul Jothiraman and Kedarnath N Sastry

Biocon Limited
Bangalore, India.

Trypsin is a highly valuable protease that has many industrial and biomedical applications. The proteolytic and autocatalytic activity of trypsin makes it very difficult to express it recombinantly. The growing demand for non-animal sources of trypsin has driven the interest to clone and express this protease in *Pichia pastoris*. Here we present successful expression of Bovine trypsinogen by fusing with 97-amino acid *Rhizopus oryzae* lipase signal sequence. We expressed the fusion protein in *Pichia pastoris* GS115 strain as well as wild type isolate of *Pichia pastoris*. The presence of prolipase sequence stabilized the expression of trypsinogen and appeared to prevent activation *in vivo*. This prolipase acts as an N-terminal extension of lipase, distinct from the signal sequence which is necessary for the transport of the protein through the membrane or for its secretion into the extracellular medium. Prolipase is known to slow down the folding rate of lipase (Beer H.D., et. al., Biochem. J. 319:351-359, 1996). The prolipase sequence also enhanced the expression of Rhizopus Glucoamylase in *Pichia pastoris* by ~300 fold (unpublished data). Fermentation, followed by microfiltration and ultrafiltration of this recombinant trypsin results in homogenous protein with very good activity.

P6 Methanol free, inducible expression of porcine trypsinogen employing new promoter variants in *Pichia pastoris*

Ruth, C.[1], Zuellig, T.[3], Mellitzer, A.[1], Looser, V.[3], Kovar, K.[3] and Glieder, A.[1,2]

[1] *Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Research Centre Applied Biocatalysis, Graz, Austria*
[3] *Institute of Biotechnology, University of Applied Sciences Zürich, Waedenswil, Switzerland*

Natural tools for recombinant protein production show serious technological limitations. Available promoters for gene expression in *Pichia pastoris* are either constitutive, weak or require the use of undesirable substances or procedures for induction. Here we show the application of new *AOX1* promoter variants for strong, tightly regulated gene expression in the yeast *Pichia pastoris*, at the same time avoiding methanol for induction.
Based on sequence analysis and deletion studies we created a library of new promoter variants with new technological features. Identified positive acting elements were then added to basal promoter fragments creating new synthetic promoters.
Trypsinogen, the precursor of the serine protease trypsin, was expressed using selected synthetic promoters and deletion variants, which allow strong induction by simple derepression. Depending on the applied promoter the production window for trypsinogen changed significantly. Within a glucose fed batch phase finally 100 times more trypsinogen was produced with promoter variants, compared to the *AOX1* wild type promoter. In addition the time where autoproteolytic degradation of trypsinogen started and space time yields were altered, depending on the employed promoter variant.

P7 Sequencing and Annotating *Pichia pastoris* CBS 7435

Andreas Küberl[1,2], Gerhard Thallinger[1], Helmut Schwab[3], Harald Pichler[3]

[1] *Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria*
[2] *Research Centre Applied Biocatalysis, Graz, Austria*
[3] *Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria*

Pichia pastoris is a methylotrophic yeast of growing interest as an expression host for recombinant protein production. Until now there are two genome sequences of *Pichia pastoris* that have been published: (i) the histidine-auxotrophic strain GS115 (developed by Cregg *et al.*, 1985), (ii) the strain referred as type strain (DMSZ 70382). Both publications report a genome size of about 9.4 Mbp on 4 chromosomes. We have in addition now sequenced the strain *Pichia pastoris* CBS 7435 wild-type strain which was developed as a new host providing freedom to operate in expression work. Our assembly suggests a genome size of about 9.3 Mbp coding for about 5.000 genes – plus about 45 genes (not including tRNAs) in the mitochondrial genome. As CBS 7435 will gain an important role as host in industrial biotechnology applications we expect big leaps forward in studying metabolic pathways and analyses of the expression and regulation systems. The genome sequence of this strain will provide a strong basis for rapid development of efficient cell factories for protein and metabolite production and for whole cell biocatalysis.

P8 Advancements in *Pichia pastoris* expression platform: improving targeting efficiency for the introduction of new auxotrophies

Laura Näätsaari[1], Beate Pscheidt[2], Claudia Ruth[1], Sandra Abad[2], Kerstin Kitz[2], Stefan Ertl[1], Clemens Mayer[1], Viktorija Vidimce[1], Roland Weis[3], Anton Glieder[1]

[1]*Department of Molecular Biotechnology, Graz University of Technology, Austria*
[2]*Research Centre Applied Biocatalysis, Graz, Austria*
[3]*VTU Technology, Grambach, Austria*

Basic expression systems for recombinant protein production in *Pichia pastoris* have been commercially available in the recent past. We have developed a new independent and well characterized expression platform with improved vectors and production strains. The versatility of this expression system enables the production of a wide variety of proteins with different requirements concerning promoter efficiency, expression cassette location, choice of markers or co-expression of chaperones.

To illustrate the potential of the improved platform in protein and strain engineering, we have successfully utilized the high targeting efficiency of a *P. pastoris* Δ*Ku70* strain to screen essential metabolic routes in search for new auxotrophies to be used as selection markers free of antibiotics. For the biopharmaceutical environment avoiding the use of antibiotic resistant strains, introduction and combination of new auxotrophies enables safe co-expression of multiple proteins.

P9 Expression and characterization of cytochrome P450 2D6 variants in *Pichia pastoris*

Geier, M.[1], Marienhagen, J.[2], Schwaneberg, U.[2], and Glieder, A.[1]

[1]*Institute of Molecular Biotechnology, Graz University, Graz, Austria*
[2]*Department of Biotechnology, RWT, Aachen, Germany*

Cytochrome P450s catalyze the introduction of a single atom of molecular oxygen to a nonactivated carbon atom of substrates, which is difficult to perform by standard chemical means. This turns them into very interesting candidates for industrial application in biotechnology and metabolite synthesis for pharmacological studies. Nevertheless, the lack of simple high-throughput expression and screening methods hinders the development of improved P450 biocatalysts from eukaryotes.
Here we show the use of the efficient expression host *P.pastoris* for laboratory evolution of cytochrome P450s.
The current work is focused on improving the catalytic properties of the cytochrome P450 2D6 (CYP2D6) towards testosterone, an atypical CYP2D6 substrate. Site saturation mutagenesis was performed on two positions believed to play a role in substrate specificity. Whole cell conversions of testosterone coupled with a fluorimetric assay as well as coupled with HPLC-MS constitutes a rapid screening system to evaluate the potential of the created CYP2D6 variants.

P10 An engineering approach for online monitoring of dynamics in protein expression of *Pichia pastoris* based on PAT-applications and mathematical process description

R. Luttmann, L. Hukelmann, K. Lögering, A. Kazemi, G. Cornelissen
Research Center of Bioprocess Engineering and Analytical Techniques
HAW – Hamburg University of Applied Sciences, Hamburg, Germany

Highly instrumented research bioreactors with a series of additional inline probes and atline analytical systems were used for analysis of dynamic processes for production of recombinant chemokines with *Pichia pastoris*.

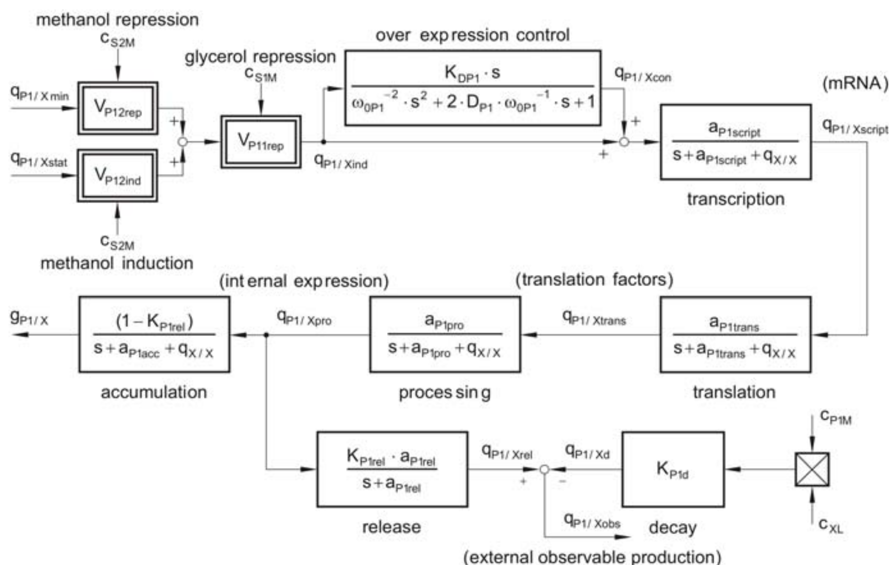
Due to the HPLC atline measurements of the substrate glycerol (S1) and the product 1-3del I-TAC (Interferon-inducible T-cell -chemoattractant, P1), inline measurements like turbidity (optek-Danulat), conductance and capacitance (ABER Instruments) and the methanol concentration (S2) in the broth as well as online off gas analysis, a completely observation of the cell density and cell viability together with all important volumetric supply and disposal rates as well as cell specific reaction rates ($q_{X/X}$, $q_{S1/X}$, $q_{S2/X}$, $q_{O/X}$ and $q_{P1/X}$) were realized online.

In combination with offline measurements of the cell specific target protein level ($g_{P1/X}$) and AOX level ($g_{P2/X}$) these data build the basis of creating a cybernetic model for the description of cell internal expression, the resulting protein secretion and the degradation of target protein in the broth.

The formation of AOX and target protein is described with a series of molecular and protein processing delay steps. The observed transient over expression controlled by the strong promoter P_{AOX1} is modelled with a critical damped disturbance transfer behavior.

The arranged model, adapted with process data, offers interesting insights in the dynamic expression and secretion processes and enables an online process observation.

It is furthermore the basis for the development of fully automated processes for the production of technical enzymes and potential vaccines candidates against malaria.



Block diagram of a Cybernetic Model for recombinant target protein expression

P11 Production and purification of artificial malaria vaccines from high cell density cultivations with *Pichia pastoris*

S. Martens[1], B. Faber[2], S.-O. Borchert[1], P. Hartwich[1], G. Cornelissen[1], B. Anspach[1], R. Luttmann[1]

[1] *Research Center of Bioprocess Engineering and Analytical Techniques*
HAW – Hamburg University of Applied Sciences, Hamburg, Germany
[2] *Biomedical Primate Research Center, Department of Parasitology*
Rijswijk,The Netherlands

A malaria vaccine which prevents or reduces infection and minimizes morbidity and mortality is a very useful additional tool for control and prevention programs against the disease. Synthetic genes with a mix of fused designed diversity covering *Plasmodium falciparum* Apical Membrane Antigen 1 (PfAMA1) and a modified form of the 19kDa c-terminal fragment of Merozoite Surface Protein 1 ($PfMSP1_{19}$) were constructed for providing a broader functional immunity and used to transform the methylotrophic yeast *Pichia pastoris*, allowing recombinant expression.[2]

However, these pharmaceutical products are obliged to meet great demands regarding protein processing in a bioreactor and in the following purification steps.
A highly instrumented bioreactor BIOSTAT® ED5 (Sartorius Stedim Systems GmbH) was configured for repeated fed batch cultivations and provided with an atline monitoring of the target protein production via HPLC.
The mainpart of complexity and costs is formed by the essential avoiding and/or removal of byproducts in a harmless way hence a minimum of purification steps is strived.
Expanded bed chromatography as an integrated method combines particle separation, product concentration and partial protein purification in one method and thus has found widespread applications in the purification of proteins from microbial sources in Industrial Bioprocessing.
Approaches for the integration of an Expanded Bed Absorption for an Integrated Bioprocess were performed and thus a fully automated production of artificial malaria vaccines was done.

Starting with the development of fully automated multi-stage production with standard bioreactors where PAT applications are introduced in research and production reactors (high instrumentation).
Based on recombinant protein and glycerol detection via atline process-HPLC's and on cell density plus cell viability inline measurements volumetric and cell specific reaction rates were calculated and observed quasi online.

The study of resulting expression dynamics leads to a proper mathematical description and to concepts of Integrated Bioprocessing. This yields in industrial developments of appropriate equipment with Sartorius Stedim Systems and GEA Westfalia Separator.

First a new reactor concept was realized for a repeated fed batch protein production in the bioreactor and integrated simultaneous protein separation steps via cross flow filtration, which are operated sequentially.

Another example is the development of a fully automated production strategy in a scale-down production plant for repeated high cell density cultivations and sequentially integrated cell separations with a new developed separator SC1 and additional protein purification procedures with an ÄKTApurifier 100 chromatography system. At both plants the recombinant protein was measured at three different places.

Applications of Design of Experiment (DoE) enable post experimental identification of optimal expression parameters. Screening and optimization runs are carried out in one bioreactor (sequential DoE) as well as in a 6 fold multifermenter system with a seventh mother fermenter for fresh cell production (sequential/parallel DoE).

Moreover atline measurements of product concentration and/or product quality and a modern automation structure opens a wide field of online optimization in the recombinant protein production plants by application of genetic algorithm or the simplex strategy from Nelder/Mead.

P12 Optimization of recombinant enzyme production with *Pichia pastoris* in a fully automated integrated scale down production plant

K. Lögering, J. Fricke, C. Müller, H.-P. Bertelsen, U. Scheffler, R. Luttmann

Research Center of Bioprocess Engineering and Analytical Techniques
HAW – Hamburg University of Applied Sciences, Hamburg, Germany

The production of the recombinant technical enzyme Lipase B from *Candida antarctica* (CALB) with *Pichia pastoris* is used as an example for a bioprocess development. The strategy and the setup of a fully automated scale down version of an Integrated Bioprocess will be illustrated first. It contains the following subsystems: A bioreactor BIOSTAT® ED10 for cell growth, a production bioreactor BIOSTAT® C30, a separator SC1 and a cross flow filtration unit Sartocon Slice.

The process strategy occurs in two parallel repeated fed batch cultivations with different substrates. In the first bioreactor glycerol is fed initially to grow up cell mass at a maximum specific growth rate and then at a certain cell density the adaption of the metabolism for production is induced with a methanol feed. For the following production with an intense reduced lag phase due to this strategy where methanol is still used for the expression of CALB the cells are transferred into the production bioreactor. The harvest is performed in cyclic separations of cells from product containing supernatant and a following clearance of residual cells from the supernatant by a cross flow filtration.

Such process guiding requires a proper timing of single process steps and has a high potential for an optimization of the production within the cultivation strategy, where in a continuously process a series of production cycles can be performed independently.

Design of Experiments (DoE) was used to find optimal cultivation parameters as well as an optimal production and harvest coordination. This article includes an illustration of the plant concept, the development of optimal process strategies, the atline detection of CALB concentration and activity as well as the global automation of such processes.

P13 Conception of a sequential/parallel DoE-design of experiments in a multifermenter system BIOSTAT® Q*plus*6 for optimal recombinant malaria vaccine production

J. Fricke[1], F. Tatge[1], H.-P. Bertelsen[1], U. Scheffler[1], B. Faber[2], R. Luttmann[1]

[1] *Research Center of Bioprocess Engineering and Analytical Techniques*
HAW – Hamburg University of Applied Sciences, Hamburg, Germany
[2] *Biomedical Primate Research Center, Department of Parasitology,*
Rijswijk, The Netherlands

The development of an optimal Active Pharmaceutical Ingredient production implies an excellent knowledge of the whole bioprocess and automation structure. This leads to a search of an optimum in a multi-parameter domain with an unknown mathematical relationship between the performance index, e.g. the volumetric protein expression productivity, and the associated control variables of upstream and/or downstream unit operations of a bioprocess. The design of a fully automated sequential/parallel procedure for a multi-parameter DoE is explained in this contribution.

For a sequential supply of a sixfold BIOSTAT® Q*plus* – 1 L system with fresh vital cell broth, a 5 L BIOSTAT® B*plus* as a motherfermenter is used. A DoE with different parameter settings can be operate in parallel for each vessel. Additional simultaneous DoE-cultivations with the same inoculation conditions, but new parameter settings, are initialized after a total release of broth and cells.

The development of a highly instrumented multibioreactor system and fully automated DoE-procedures will be explained. Furthermore first results in optimizing the expression conditions for the production of a promising malaria vaccine candidate, the *Plasmodium falciparum* Apical Membrane Antigen 1 (PfAMA-1), with *Pichia pastoris* will be described. For a fast quasi online detection of the target protein first trials with atline applications of LOV-LabOnValve techniques will be discussed.

P14 Expression and characterization of a pH stable *Ganoderma fornicatum* laccase in *Pichia pastoris*

Wan-Ting Huang and Ching-Tsan Huang

Institute of Microbiology and Biochemistry, National Taiwan University
Taipei, Taiwan

White-rot fungi have been seemed a good resource of laccase genes due to their high ligninolytic activity. In this study, a full-length cDNA of laccase from the medicinal white-rot fungus Ganoderma fornicatum 0814 was cloned and expression in *Pichia pastoris* KM71H. The corresponding open reading frame has 1563 nucleotides and encodes a protein of 521 amino acids including a 21-residue secretion signal peptide. The optimum pH and temperature for the recombinant laccase r0814 is pH 3.5 and 55-75°C, respectively. This recombinant laccase retained more than 85% activity after 24 h treatment at pH ranging from 2.5 to 10, and at least 81, 62 and 44% activity after 30 min treatment in 40, 60 and 80% methanol, respectively. This recombinant laccase was pH-stable and methanol-tolerant. The Km, Vmax, and Kcat / Km values of r0814 using 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) as the substrate was 0.142 mM, 0.001 mM min-1 and 10000 mM-1min -1, respectively. These properties suggest that r0814 has high potential for industrial appreciations.

P15 Optimization of the heterologous production of a *Rhizopus oryzae* lipase (ROL) in *Pichia pastoris* system using mixed substrates by controlled fed-batch bioprocess

Carolina Arnau, *Ramon Ramon, Carles Casas and Francisco Valero

Departament d'Enginyeria Química. ETSE. Universitat Autònoma de Barcelona Bellaterra (Barcelona), Spain
** Bioingenium, S.L. Edifici Hèlix. Parc Científic de Barcelona*
Barcelona, Spain

To increase cell density and process productivity, as well as to reduce the induction time, a typical approach is the use of a multicarbon substrate in addition to methanol in *Pichia pastoris* system producing heterologous proteins (Files et al. 200, Cos et al., 2007). The mixed substrate strategy applied to Mut^s ROL production fed-batch culture at a low residual methanol concentration has allowed increasing the production of heterologous protein Mut^s phenotype, avoiding the drastic reduction of the specific production rate observed after the start of the induction phase when methanol is used as sole carbon source (Ramón et al., 2007).

A systematic study of the influence of methanol set-point and the use of co-substrates (sorbitol and glycerol) feeding rates in fed-batch operation with Mut^s phenotype in heterologous ROL production using *Pichia pastoris* system are presented. Five experiments were made at a constant methanol set-point of 0.5 $g{\cdot}l^{-1}$, 2 $g{\cdot}l^{-1}$ and 4 $g{\cdot}l^{-1}$ controlling by a predictive control algorithm with two different sorbitol feeding rate to assure a constant specific rate of 0.01 h^{-1} and 0.02 h^{-1} by means of a pre-programmed exponential feeding rate strategy.

Lipolytic activity, yields, productivity and specific productivity, but also specific growth, consumption and production rates were analyzed showing that the best conditions were reached when the methanol set-point was 2 $g{\cdot}l^{-1}$ with a low influence of the constant specific growth tested.

Glycerol was also tested as a co-substrate at the optimal sorbitol conditions reaching similar ROL production. However, when the specific growth rate was $0.1h^{-1}$, lipolytic activity, yields, productivity and specific productivity were significantly lower, showing the influence of specific growth rate on ROL production when glycerol was used as co-substrate.

Acknowledgements
This work was supported by the project CTQ2007-60347 of the Spanish Ministry of Science and Innovation, 2009-SGR-281 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya).

References:
Cos *et al.,* Microbial Cell Factories 5. 2006.
Files et al., Enzyme Microb.Technol. 29[6-7], 335-340. 2001.
Ramon. et al., J. Biotechnol. 130, 39-46. 2007.

P16 Secretion and proteolysis of heterologous proteins fused to the *E. coli* maltose binding protein in *Pichia pastoris*

Zhiguo Li[#], Wilson Leung*, Amy Yon*, Vincent C. Perez*, John Nguyen*, Jane Vu*, William Giang*, Linda Luong*, Kate A. Salazar*, Tracy Phan*, Seth R. Gomez*, Andreas Franz[#], Geoff P. Lin-Cereghino*, and Joan Lin-Cereghino*

** Department of Biological Sciences, University of the Pacific*
Stockton, California
 # Department of Chemistry, University of the Pacific
Stockton, California

The *E. coli* maltose binding protein (MBP) has been utilized as a translational fusion partner to improve the expression of foreign proteins made in *E. coli*. MBP has been shown to increase the solubility of intracellular proteins as well as improve the export of secreted proteins. We initially explored whether MBP would have the same effect in *Pichia pastoris*. When MBP was fused as an N-terminal partner to several C-terminal cargo proteins expressed in this yeast, proteolysis occurred between the two peptides and only MBP reached the extracellular region. Extensive mutagenesis of the spacer region between MBP and its C-terminal cargo protein could not inhibit the cleavage although it did cause changes in the protease target site in the fusion proteins, as determined by mass spectrometry. Taken together, these results suggested that the three dimensional structure of MBP triggered attack by an uncharacterized *P. pastoris* protease at a nonspecific region C-terminal of the MBP domain. However, MBP was able to serve as a secretion enhancer when it was fused as a C-terminal peptide to an N-terminal cargo protein. These studies provide new insights into the role of proteases and fusion partners in the secretory mechanism of *P. pastoris.*

P17 *Pichia* Glycoswitch^{TM} technology: switching the N-glycosylation

Tiels P, Deschutter K, Van West E, Callewaert N

VIB
Ghent, Belgium

The methylothropic yeast *Pichia pastoris* is one of the most commonly used organisms for the production of recombinant proteins and the study of peroxisomal biogenesis and methanol assimilation. P. Pastoris combines the ability to grow on minimal medium to very high cell densities and the ability to secrete the heterologous protein with the ability to perform the higher eukaryotic post-translational modifications. The mayor disadvantage in the use of yeast in the production of biopharmaceuticals is the non-human like glycosylation, which can be immunogenic and leads to rapid clearance. Recent advantages have led to the development of yeast strains with fully humanised N-glycosylation. Here we present the *Pichia* GlycoSwitch^{TM} technology in which the yeast's own hyperglycosyl N-glycans are switched to the humanised biantennary complex type N-glycans. By disrupting the P. pastoris OCH1 gene and introducing 6 heterologous enzyme activities we succeeded to produce Gal2GlcNAc2Man3GlcNAc2-modified glycoproteins. Recent achievements in the field of P. pastoris will increase the importance of this yeast for the production of glycoproteins, as the annotation of the P. pastoris genome sequence revealed interesting features that can be used to increase productivity, homogeneity and stability of the produced glycoprotein.

P18 Glyco-systems engineering of the non-conventional yeast /Yarrowia lipolytica/ for homogenous glycoprotein modification with the universal Man3GlcNAc2 N-glycan precursor.

Karen De Pourcq[1] , Annelies Van Hecke[1] and Nico Callewaert[1,2].

[1] Department for Molecular Biomedical Research, VIB
Ghent, Belgium.
[2] Department of Biochemistry, Physiology and Microbiology, Ghent University
Ghent, Belgium.

/Yarrowia lipolytica/ is a GRAS-status dimorphic yeast that efficiently secretes various heterologous proteins. Therefore it is an attractive protein production host. However, yeasts modify glycoproteins with non-human high mannose-type N-glycans. These structures drastically reduce /in vivo/ protein half-life and may be immunogenic in man. Here, we describe how we interfered with the N-glycan biosynthesis in /Yarrowia lipolytica/, to obtain homogenous Man_3 GlcNAc_2 structures on its glycoproteins. This is the ideal starting point to build up human-like sugars.

We disrupted the /ALG3/ gene and found mainly Man_5 GlcNAc_2, GlcMan_5 GlcNAc_2 and to a lesser degree Glc_2 Man_5 GlcNAc_2 on the glycoproteins. To avoid possible glycosylationsite underoccupancy we concomitantly overexpressed /ALG6/. The terminal glucose residues hamper further humanization of the N-glycosylation. Therefore, several approaches were tested to remove them and overexpression of the /Apergillus niger/ glucosidase II proved to be the most efficient. Lastly we overexpressed an a-1,2-mannosidase to obtain Man_3 GlcNAc_2 structures which are the substrate for complex-type glycans.

The final glyco-engineered /Yarrowia lipolytica/ strain produces glycoproteins mainly containing trimannosyl core N-glycans (Man_3GlcNAc_2) which is the common backbone of various human-type N-glycans.The results demonstrate the high potential of /Yarrowia lipolytica/ to be developed as an efficient expression system for the production of glycoproteins with humanized glycans.