ABSTRACT BOOK

Pichia 2012 Conference
February 29 – March 3 2012
Alpbach, Austria
We thank our Sponsors
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Scientific Committee

David Bramhill (RCT, Tucson, Arizona, USA)
Nico Callewaert (Ghent University, Belgium)
Jim Cregg (Keck Graduate Institute of Applied Life Science, Claremont, California, USA)
Anton Glieder (ACIB GmbH, Graz, Austria)
Karin Kovar (ZHAW, Wädenswil, Switzerland)
Joan and Geoff P. Lin-Cereghino (University of the Pacific, Stockton, California, USA)
Diethard Mattanovich (University of Natural Resources, Austria)
Helmut Schwab (Graz University of Technology, Austria)

Organizational Committee

Anton Glieder (ACIB GmbH, Graz, Austria)
Tanja Hajek (ACIB GmbH, Graz, Austria)
Clemens Keil (Medical University of Graz, Austria)
Karin Kovar (ZHAW, Wädenswil, Switzerland)
Astrid Preisz (ACIB GmbH, Graz, Austria)
Venue Map

Congress Center Alpbach
Alpbach 246, 6236 Alpbach, Austria, Phone: +43 5336 600 100
Schedule at a Glance

Wednesday, February 29, 2012

4:00pm  Registration  Otto Molden Foyer
6:00pm  Welcome  Salon Schrödinger
6:15pm  Keynote Lectures  Salon Schrödinger
8:00pm  Dinner  Salon Schrödinger

Thursday, March 1, 2012

08:00am  AV check for today’s speaker  Salon Schrödinger
08:30am  Session 1: Inside of the black box  Salon Schrödinger
         Chair: Diethard Mattanovich
09:50am  Break  Otto Molden Foyer
10:15am  Session 2: Rational Design of Protein Secretion  Salon Schrödinger
         Chair: Nico Callewaert
11:35am  Poster Session 1  Otto Molden Foyer
1:00pm   Lunch Break (Lunch Package)  Otto Molden Foyer
         Individual transfer to cable car station for skiers
1:30pm   Meeting point for “rodeln” and hiking  Otto Molden Foyer
5:15pm   Bus transfer to cable car  Bus Reversal close to Conference Center
6:00pm   Dinner on Top of the Mountain  Berggasthof Hornboden
ca.9:30 pm  Transfer to Alpbach  Cable Car Station
Friday, March 2, 2012

08:00am  AV check for today’s speaker  
Salon Schrödinger

08:30am  **Session 3: Manipulation of Post-translational Modifications**  
Chair: Roland Weis  
Salon Schrödinger

09:50am  **Break**  
Otto Molden Foyer

10:20am  **Session 4: Bioprocess Design**  
Chair: Pau Ferrer  
Salon Schrödinger

12:00pm  **Lunch Break & Poster Session 2**  
Kunst Foyer and Salon Lichtenstein & VonHayek

1:30pm  **Session 5: Why Methanol?**  
Chair: Jim Cregg  
Salon Schrödinger

3:30pm  **Break**  
Otto Molden Foyer

4:00pm  **Session 6: Enzymes and Whole Cell Catalysts**  
Chair: Helmut Schwab  
Salon Schrödinger

6:00pm  **Break & Poster Session 3**  
Otto Molden Foyer

8:00pm  **Event Dinner at Congress Center & Sandoz Poster Price Awarding**  
Salon Schrödinger

Saturday, March 3, 2012

07:30-9:00  **Breakfast in Conference Center**  
Kunst Foyer and Salon Lichtenstein & VonHayek

08:30am  AV check for today’s speaker  
Salon Schrödinger

09:00am  **Session 7: New Pharma Proteins**  
Chair: Kurt Gehlsen  
Salon Schrödinger

10:45am  **Break (Removal of posters)**  
Otto Molden Foyer

11:15am  **Session 8: New Concepts and Tools**  
Chair: Günther Daum  
Salon Schrödinger

1:00pm  **Conference close. Pick up lunch**  
Otto Molden Foyer
Pichia 2012
PROGRAM
Wednesday, February 29, 2012

4:00pm  
*Registration*

6:00pm  
Welcome
Toni Glieder
ACIB GmbH, Austria

6:15pm  
Keynote Lectures
Chair: Karin Kovar

T1: Development of the yeast *Pichia pastoris* as a recombinant protein production host
Jim Cregg
Keck Graduate Institute of Applied Life Science, USA

7:00pm  
T2: *Pichia pastoris* - Black box or law and order
Guenter Daum
Graz University of Technology, Austria

8:00pm  
Dinner

* Posters should be fixed during registration hours, poster walls will be marked with your poster number according to the poster session overview (see page 15)
Thursday, March 1, 2012

08:30am  **Session 1: Inside of the black box**  
Chair: Diethard Mattanovich

T3: Refined genome scale model of *Pichia pastoris*  
Jürgen Zanghellini  
ACIB GmbH, Austria

08:50am  **T4:** Integration of transcriptomic and metabolomic data reveals distinct traits in the metabolic regulation of recombinant *Pichia pastoris'* central carbon metabolism  
Marc Carnicer  
Universitat Autònoma de Barcelona, Spain

09:10am  **T5:** Analyzing the early secretory pathway in *Pichia*  
Ben Glick  
University of Chicago, USA

09:50am  *Break*

10:15am  **Session 2: Rational Design of Protein Secretion**  
Chair: Nico Callewaert

T6: Predicting protein secretion from *Pichia* based on protein stability  
David Archer  
University of Nottingham, UK

10:55am  **T7:** Reverse engineering of protein secretion by uncoupling of cell cycle phases from growth  
Markus Buchetics  
University of Natural Resources and Life Sciences, Austria

11:15am  **T8:** Engineering of protein folding & secretion in *Pichia pastoris* - A systems biology approach  
Brigitte Gasser  
University of Natural Resources and Life Sciences, Austria

11:35am  **Poster Session 1**

1:00pm  *Lunch Break (Lunch Package)*  
Possibility for skiing, „rodeln“ and other funny outdoor activities

6:00pm  *Dinner on Top of the Mountain*
**Friday, March 2, 2012**

08:30am  **Session 3: Manipulation of Posttranslational Modifications**  
Chair: Roland Weis

  **T9:** Genome resequencing to find mutations and as a tool for glycoengineering  
Nico Callewaert  
Ghent University, Belgium

09:10am  **T10:** Get product quality under control! Factors affecting the variability of heterologous proteins during production processes with recombinant *P. pastoris*  
Shaikh Rafeek  
Zurich University of Applied Sciences, Switzerland

09:30am  **T11:** Improving the biophysical properties of full-length IgGs by using *Pichia pastoris* as expression host: lessons from the comparison of the yeast production system with mammalian cell expression  
Jonas V. Schaefer  
University of Zurich, Switzerland

09:50am  **Break**

10:20am  **Session 4: Bioprocess Design**  
Chair: Pau Ferrer

  **T12:** Strategy development for therapeutic protein production by *Pichia pastoris*  
Pinar Calik  
Middle East Technical University, Turkey

11:00am  **T13:** Development of an optimized sequential production and purification process for recombinant proteins with *Pichia pastoris* in a fully automated integrated pilot plant  
Christian Müller  
Hamburg University of Applied Sciences, Germany

11:20am  **T14:** Optimization of a sequential pharmaceutical protein production process with in-situ product removal (ISPR)  
Gesine Cornelissen  
Hamburg University of Applied Sciences, Germany
11:40am  **T15:** A systems and synthetic biology framework for pathway level culture media engineering: application to *Pichia pastoris*  
Rui Oliveira  
Universidade Nova de Lisboa, Portugal

12:00pm  *Lunch Break + Poster Session 2*

1:30pm  **Session 5: Why Methanol?**  
Chair: Jim Cregg

**T16:** Regulation of catabolite repression of AOX1 promoter by Mig in *Pichia pastoris*  
Xiangshan Zhou  
East China University of Science and Technology, P.R. China

2:00pm  **T17:** Doing the splits – yielding g/L levels with methanol-driven and methanol-free production from VTU’s AOX1 promoter library  
Roland Weis  
VTU Technology GmbH, Austria

2:30pm  **T18:** Purification of AOX1 promoter-binding proteins from *Pichia pastoris*  
Mehmet Inan  
Akdeniz Universiy, Turkey

2:50pm  **T19:** Playing with the methanol utilization pathway in *Pichia pastoris*  
Oliver Spadiut  
Vienna University of Technology, Austria

3:10pm  **T20:** Higher titres with less methanol – cultivation of *Pichia pastoris* Mut' with mixed substrates  
Leona Paulová  
Institute of Chemical Technology Prague, Czech Republic

3:30pm  *Break*

4:00pm  **Session 6: Enzymes and Whole Cell Catalysts**  
Chair: Helmut Schwab

**T21:** Application of fed-batch screening at microscale and scale-up to laboratory fermenter with a lipase expressing *Pichia pastoris* strain  
Pau Ferrer  
Universitat Autònoma de Barcelona, Spain

4:40pm  **T22:** Making synthetic proteins by *Pichia pastoris*  
Birgit Wiltschi  
ACIB GmbH, Austria
5:00pm  **T23:** *Pichia pastoris* as whole-cell biocatalyst for (+)-Nootkatone production  
Harald Pichler  
Graz University of Technology, Austria

5:20pm  **T24:** Commercializing the best enzymes from nature  
David Weiner  
Verenium Corporation, USA

6:00pm  *Break & Poster Session 3*

8:00pm  *Event Dinner at Congress Center & Sandoz Poster Price Awarding*
Saturday, March 3, 2012

07:30-9:00  Breakfast in Conference Center

09:00am  Session 7: New Pharma Proteins  
Chair: Kurt Gehlsen

09:05am  25: Nanobodies from bench to bedside, with a big help of *Pichia*  
Peter Schotte  
Ablynx NV, Belgium

09:35am  T26: High throughput methods for *Pichia pastoris* pave the way for rapid process development - Case study: Production of pharmaproteins fused to human serum albumin as half-life extension  
Simon Stammen  
Boehringer Ingelheim RCV GmbH & Co KG, Austria

10:05am  T27: Analyses of a Monoclonal Antibody Produced by *Pichia pastoris*  
Satoru Misawa  
API Corporation, Japan

10:25am  T28: Production of a malaria transmission blocking vaccine candidate Pfs25 in *Pichia pastoris* for human clinical studies  
Nicholas MacDonald  
Laboratory of Malaria Immunology and Vaccinology, USA

10:45am  Break (Removal of posters)

11:15am  Session 8: New Concepts and Tools  
Chair: Günther Daum

T29: New strategies for induction and screening of high expressing clones  
Helmut Schwab  
Graz University of Technology, Austria

11:35am  T30: Characterization of recombinant protein production in *Pichia pastoris* under control of the novel THI11 promoter and design of a tailor-made production process  
Michael Maurer  
University of Natural Resources and Life Sciences, Austria
11:55am  **T31**: Production of recombinant proteins under oxygen limited process conditions: scFv production, a case study
Narendar Kumar Khatri
University of Oulu, Finland

12:15am  **T32**: Enzyme-based Glucose Delivery System Enhances Recombinant Protein Production in Shaken Cultures of *Pichia pastoris*
Antti Vasala
BioSilta Oy, Finland

12:35am  **T33**: Alternative secretion signals for *Pichia pastoris*
Andrea Camattari
Graz University of Technology, Austria

12:55am  Closing Remarks
Toni Glieder
ACIB GmbH, Austria

1:00pm  *Conference close. Pick up lunch*
Poster Session
Overview
P1 Optimization of methanol-free fermentation with 2nd generation AOX1 promoter variants
Atlic A., Plank H., Kern A., Bona R., Dib I., Purkarthofer T., Weis R.
VTU Technology GmbH, Parkring 18, 8074 Grambach/Graz, Austria

P2 Clonal Variation in recombinant Pichia pastoris
Aw R. (1), Kara B. (2), Leak D.J. (1)
(1) Imperial College London, London, UK
(2) Fujifilm Diosynth Biotechnologies, Billingham, UK

P3 Trick or treat – optimizing adaptation of Pichia pastoris to methanol in fermentation
VTU Technology GmbH, Parkring 18, 8074 Grambach/Graz, Austria

P4 Whole-cell screening of functional CYP52s and reductases for heterologous expression in Pichia pastoris
Camattari A. (1), Gudiminchi R. (1), Lanfranchi E. (1) and Glieder A. (2)
(1) Institute of Molecular Biotechnology, Technical University of Graz, Petersgasse 14/2, Graz, Austria
(2) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14/5, Graz, Austria

P5 A multistep strategy for selection of high diabody producers in P. pastoris.
(1) Eurogentec S.A., New Technology Development, 5, rue Bois Saint Jean, 4102 Seraing, Belgium
(2) Eurogentec S.A., Process Transfer & Development, 5, rue Bois Saint Jean, 4102 Seraing, Belgium

P6 Expression of a Trichoderma reesei endo-N-acetyl-β-D-glucosaminidase (EndoT) in Pichia pastoris
De Schutter K. (1,2), De Pourcq K. (1,2), Laukens B. (1,3), and Callewaert N. (1,3)
(1) Unit for Medical Biotechnology, Department for Molecular Biomedical Research, VIB, Technologiepark 927, 9052 Ghent-Zwijnaarde, Belgium.
(2) Department for Biomedical Molecular Biology, Ghent University, Technologiepark 927, 9052 Ghent-Zwijnaarde, Belgium.
(3) Unit for Molecular Glycobiology, L-ProBE, Department for Biochemistry and Microbiology, Ghent University, Ledeganckstraat 35, 9000 Ghent, Belgium.

P7 Expression and glyco-engineering of Alpha-galactosidaseA in Pichia pastoris for the treatment of Fabry disease
De Visscher C. (1,2), Tiels P. (1,2), Vanhecke A. (1,2), Callewaert N. (1,2)
(1) Unit for Medical Biotechnology, Department for Molecular Biomedical Research, VIB, Ghent, Belgium
(2) Laboratory for Protein biochemistry and Biomolecular Engineering (L-ProBE), Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium.

P8 Maximizing Space-Time-Yield with 2nd Generation AOX1 Promoter Variants
VTU Technology GmbH, Parkring 18, 8074 Grambach/Graz, Austria

P9 From Strain to Product: A fast approach in bioprocess development for recombinant protein expression in Pichia pastoris
Dietzsch C., Spaduti O., Zalai D., Herwig C.
Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria
P10 Improved processing of secretory proteins in *Pichia angusta* by sequence variation of the alpha mating factor prepro sequence
Eilert E. (1), Rolf T. (1), Hollenberg
C.P. (2), Piontek M. (1), Suckow M. (1)
(1) ARTES Biotechnology GmbH, Elisabeth-Selbert-Str. 9, Langenfeld (Rheinland), Germany
(2) Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, Düsseldorf, Germany

P11 *Pichia pastoris* as cell factory – Examining the secretory capabilities
Felber M. (1,2), Buchetics M. (1,3), Gasser B. (1,3), Schwab H. (1,2), Pichler H. (1,2)
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, 8010 Graz, Austria
(2) Institute of Molecular Biotechnology, Graz University of Technology, 8010 Graz, Austria
(3) Institut für angewandte Mikrobiologie, BOKU Vienna, 1190 Wien, Austria

P12 100% double site saturation by individual expression of 400 muteins in *P. pastoris*
Geier M. (1), Braun A. (1), Glieder A. (2)
(1) Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria
(2) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, 8010 Graz, Austria

P13 Characterization of *Pichia pastoris* Golgi and plasma membrane
Grillitsch K.(1), Spanova M.(2), Zellnig G. (3.), Daum G. (1,2)
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), c/o Institute of Biochemistry, Petersgasse 14, A-8010 Graz
(2) Institute of Biochemistry, Petersgasse 12/2, A-8010 Graz
(3) Institute of Plant Sciences, Schubertstrasse 51, A-8010 Graz

P14 Advanced bioprocess monitoring by implementation of Proton Transfer Reaction - Mass Spectrometry (PTR-MS) for measurement of volatile components in the bioreactor
Gutmann R.(1,2), Luchner M.(1), Herbig J.(2), Armin H.(3) Bayer K.(1), Striedner C.(1,4)
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Muthgasse 11, 1190 Vienna, Austria
(2) Ionimed Analytik GmbH, Eduard-Bodem-Gasse 3, 6020 Innsbruck, Austria
(3) Institute of Ion Physics and Applied Physics, University of Innsbruck, Technikerstr.25/3, 6020 Innsbruck, Austria
(4) Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria

P15 *Pichia pastoris* lipid droplet lipidome
Ivashov V. (1), Grillitsch K. (1, 2), Koeferl H. (3), Leitner E.(1),Daum G.(1)
(1) Graz University of Technology
(2) Austrian Centre of Industrial Biotechnology (ACIB GmbH)
(3) Core Facility for Mass Spectrometry/Lipidomics, Center for Medical Research, Medical University of Graz, Austria

P16 Engineering *Pichia stipitis* for xylitol production from the diluted acid extract of empty palm fruit bunch fiber
Kim S., Park J. M., Seo J.W., Kim C. H.
Jeonbuk Branch Institute, Korea Research Institute of Bioscience and Biotechnology, 181 Ipsinggil, Jeongeup 580-185, Korea
P17  Lipidome of subcellular fractions from the yeast *Pichia pastoris*
Klug L. (1), Grillitsch K. (2) and Daum G. (1)
(1) Institute of Biochemistry, TU Graz, Petersgasse 12/II, 8010 Graz, Austria
(2) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, 8010 Graz, Austria

P18  Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway
(1) Graz University of Technology, Institute of Molecular Biotechnology, Graz, Austria
(2) (4) (5) Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria
(3) (6) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Graz, Austria

P19  New expression systems for heterologous protein production in *Pichia jadinii* and *Candida utilis*
Kunigo M., Bürth C., Ernst J. F.
Molecular Mycology, Heinrich-Heine-University, Duesseldorf, Universitaetsstrasse 1, 40225 Duesseldorf, Germany

P20  Bulk expression of a potential anti-HIV1 microbicide? *Pichia pastoris*, the right tool for the job
(1) Unit for Medical Biotechnology, Department for Molecular Biomedical Research, VIB-UGent, Technologipark 927, B-9052 Ghent-Zwijnaarde, Belgium.
(2) Biological Process Development Facility, Department of Chemical and Biomolecular Engineering, 304 Othmer Hall, University of Nebraska, Lincoln, Nebraska 68588 United States of America
(3) Mintaka Foundation for Medical Research, 1 route de Pré-Marais, 1233 Bernex, Switzerland
(4) Department of Structural Biology and Bioinformatics, Faculty of Medicine, University of Geneva, Geneva, Switzerland
(5) LifeSci Partners, LLC, 4211 NE 131st Street, Vancouver, Washington 98686-2811, United States of America

P21  Combined use of flow cytometry to quantify the physiological state of *P. pastoris* in high-cell-density fedbatch cultures
Hyka P.(1,2), Looser V.(1), Ruth C.(3), Meier C.(1), Melzoch K.(2), Meyer H.P.(4), Glieder A.(3), and Kovar K.(1)
(1) Institute of Biotechnology, Zurich University of Applied Sciences (ZHAW), Campus Grüental, CH-8820 Wädenswil, Switzerland, Contact: koka@zhaw.ch
(2) Institute of Chemical Technology Prague, Department of Fermentation Chemistry and Bioengineering, Technická 5, CZ-166 28 Prague 6, Czech Republic
(3) Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, A-8010 Graz, Austria
(4) Lonza Ltd., CH-3930 Visp, Switzerland

P22  PEGylate the smarter way: site-specific PEGylation of a single-domain antibody (sdAb) via chemical cleavage of intein-fusion proteins secreted from *Pichia pastoris*
Marques A. (1), Mysliwy J. (1), Cotton G. (1), Dib I. (2), Gruber U. (2), Plank H. (2), Purkarthofer T. (2) and Weis R. (2)
(1) Almac Group, Elvingston Science Centre, Edinburgh, East Lothian, UK, EH33 1EH
(2) VTU Technology, Parkring 18, Grambach, 8074 Austria
P23  An efficient approach to the design of processes for enzyme production with recombinant Pichia pastoris
Looser V., Meier C., Lüthy D., Hyka P., and Kovar K.
Institute of Biotechnology, Zurich University of Applied Sciences (ZHAW), Campus Grüental, CH-8820 Wädenswil, Switzerland, Contact: koka@zhaw.ch

P24  High level protein expression in Pichia pastoris combining synthetic promoters and synthetic genes
Mellitzer A. (1), Ruth C. (1), Weis R.(2), Glieder A. (1)
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), c/o IMBT TUGraz, Graz, Austria;
(2) VTU Technology GmbH, Grambach, Austria;

P25  Optimization of diploid Pichia pastoris strains for the expression of full-length monoclonal antibodies
(1) Alder Biopharmaceuticals, Bothell, WA
(2) Akdeniz University, Antalya, Turkey

P26  Dynamic metabolic modelling of Pichia pastoris cultures on glycerol based medium in bioreactor
Niu H. (1), Rodriguez Ch. (2), Daukandt M. (2), Fickers P. (1)
(1) Unité de Biotechnologies et Bioprocédés, Université libre de Bruxelles, Av. F.-D. Roosevelt 50 CP 165/61, 1050 Brussels, Belgium
(2) Eurogentec S.A., Rue du Bois Saint-Jean, 5, 4102 Seraing, Belgium.

P27  Identification and characterization of novel promoters in Pichia pastoris
Prielhofer R. (1), Maurer M. (2, 3), Mattanovich D. (1, 3), Gasser B. (1,3)
(1) Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
(2) School of Bioengineering, University of Applied Sciences FH Campus Wien, Vienna, Austria
(3) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Vienna, Austria

P28  Pichia and HSA (fusions) – a love story
Plank H., Bona R., Dib I., Gruber U., Purkarthofer T. and Weis R.
VTU Technology, Parkring 18, 8074 Grambach, Austria

P29  Fluorescence Microscopy of Pichia pastoris: Revealing Bottlenecks in Recombinant Protein Secretion
Puxbaum V. (1), Ertl S. (1), Gasser B. (1,2), Maccani A. (1), Kunert R. (1,2), Mattanovich D. (1,2)
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Vienna, Austria
(2) University of Natural Resources and Life Sciences, Department of Biotechnology, Vienna, Austria

P30  A new latex agglutination test for surra based on recombinant Trypanosoma evansi RoTat 1.2 variant surface glycoprotein expressed in the Pichia pastoris GlycoSwitch™ M5 strain
Rogé S.(1,3), Vervecken W.(2), Guisez Y.(3), Büscher P.(1)
(1) Institute of Tropical Medicine, Department of Biomedical Sciences, Nationalestraat 155, 2000 Antwerp, Belgium.
(2) Oxyrane Belgium nv, Technologiepark 3, B-9052 Ghent-Zwijnaarde, Belgium.
(3) University of Antwerp, Laboratory for Molecular Plant Physiology and Biotechnology, Department of Biology, Groenenborgerlaan 171, B-2020 Antwerp, Belgium.
P31 Identification and isolation of GPI anchored proteins from *Pichia pastoris* and their potential for surface display of recombinant proteins  
Rosales Rodriguez I. (1), Ahmad M. (1), Nudl T. (2), Pichler H. (1, 3), Schwab H. (1, 3)  
(1) Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria  
(2) Baxter AG, Industriestraße 67, 1221 Vienna, Austria  
(3) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, 8010 Graz, Austria

P32 Improving the biophysical properties of full-length IgG – inspired by *Pichia pastoris*  
Schaefer J. V. and Plückthun A.  
Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

P33 Production of glucoamylase enzyme variants in *Pichia pastoris* expression systems  
Tóth A.(1), Laczi K.(2), Rákhely G.(3.), Kovács KL.(4)  
(1) Institute of Biophysics, Biological Research Centre, Szeged, Hungary  
(2) (3) (4) Department of Biotechnology, University of Szeged, Szeged, Hungary

P34 UNic™ Translation Enhancement Elements  
Vottero E., Verhaert R.M.D. B., Schut V.  

P35 Production and Processing of Interferon in *Pichia* - A Cut Above  
Dib I., Gruber U., Plank H., Bona R., Purkarthofer T., and Weis R.  
VTU Technology, Parkring 18, 8074 Grambach, Austria

P36 Making synthetic proteins in *Pichia pastoris*  
Wiltschi B. (1), Wenger W. (2), Budisa N. (2)  
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, 8010 Graz  
(2) Institut für Chemie, Technische Universität Berlin, Franklinstraße 29, 10587 Berlin

P37 Random mutagenesis in *Pichia pastoris* by resistance cassette transformation  
Winkler, C., Pichler H., Schwab H.  
Institute of Molecular Biotechnology, Graz University of Technology, Austria

P38 (+)-Nootkatone production in yeasts  
Wriessnegger T. (1), Augustin P. (1), Emmerstorfer A. (1), Müller M. (2), Kaluzna I. (2), Macheroux P. (1,3), Schwab H. (1,4), Pichler H. (1,4)  
(1) Austrian Centre of Industrial Biotechnology (ACIB GmbH), Graz, Austria  
(2) DSM Innovative Synthesis B.V., Geleen, The Netherlands  
(3) Institute of Biochemistry, Graz University of Technology, Austria  
(4) Institute of Molecular Biotechnology, Graz University of Technology, Austria
Oral Presentation

Abstracts
Development of the Yeast *Pichia pastoris* as a Recombinant Protein Production Host

Cregg J (1), Tolstorukov I. (2)

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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 such as proper folding, proteolytic processing, disulfide bridge formation and glycosylation. Relative to *Saccharomyces cerevisiae*, *Pichia pastoris* has two significant physiological advantages as a host for the production of recombinant proteins. The first is the promoter used to transcribe most foreign genes, which is typically 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. The development and features of *P. pastoris* expression will be reviewed along with recent advances in the system. In addition, as an example of the utility of the system, the expression of a full length humanized monoclonal antibody will be presented.
Pichia pastoris: Black Box or Law and Order

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The yeast *Pichia pastoris* is widely used for industrial purposes, especially for the expression and production of recombinant heterologous proteins. Due to the ease of handling by nutritional and molecular means *Pichia pastoris* is used as a tool comparable to *Escherichia coli* or the baker’s yeast *Saccharomyces cerevisiae*. Despite the importance of *Pichia pastoris* in biotechnology, little information about the cell biology of this microorganism is available. Surprisingly, intracellular processes related to subcellular organization and interaction of organelles are frequently ignored. Of course, *Pichia pastoris* like all other cell types is not a Black Box where magic processes take place but a well-organized and complex machinery which allows controlled and controllable product formation. To shed more light on the cell biology of *Pichia pastoris* we started a fundamental project some years ago aimed at characterization of different *Pichia pastoris* organelles with emphasis on subcellular biological membranes and lipids. In the course of these investigations we first got some insight into structure, function and biochemical properties of mitochondria and peroxisomes. More recently, we focussed on subcellular fractions involved in the secretory pathway such as the endoplasmic reticulum (microsomes), the Golgi apparatus and the plasma membrane. As a special organelle, lipid droplets were investigated which are storage sites for non-polar lipids. For all these organelles isolation protocols were established, standard methods of protein and lipid analyses were employed, and proteome and lipidome studies were performed. Microscopic analyses supplemented these studies and provided information of the internal cell structure under different growth conditions. Similarities of *Pichia pastoris* and *Saccharomyces cerevisiae* became evident on one hand, but on the other hand specific differences between these two microorganisms have to be kept in mind. The more detailed understanding of *Pichia pastoris* cell biology will provide a solid basis for designing new and improved strategies to utilize *Pichia pastoris* for industrial applications.
Designing the most efficient producer

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Glycerol is a waste product in biodiesel production. Recently *Pichia Pastoris* has been engineered for the production of propylene glycol from glycerol. However, the production yield was poor. Based on elementary mode analysis (EMA) we present a novel computational method to predict the most efficient producer.

EMA is ideally suited for metabolic engineering, as it allows for an unbiased decomposition of metabolic networks in biologically meaningful elementary modes. By systematically deleting undesirable EM networks of minimal functionality can be generated. Our method allows for rational strain design, which identifies all sets of knockouts that result in the deletion of all undesired EM. More importantly, our methods guarantees that the minimum number of deletions characterizes these sets.

References

Integration of transcriptomic and metabolomic data reveals distinct traits in the metabolic regulation of recombinant *Pichia pastoris*' central carbon metabolism


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Global quantitative metabolome analysis provides a unique platform to characterize in a systematic way the impact of environmental and genetic perturbations on the cell’s physiology, also enabling to assess the role of different levels of regulation on the overall metabolic phenotype of an organism. Recently, the methodological basis for metabolomics for *Pichia pastoris* have been developed allowing an accurate, reproducible and reliable intracellular metabolite level quantification [1].

Recently, we identified a beneficial effect of low oxygen availability on recombinant protein production in *Pichia pastoris* [2]. In the present study, a quantitative metabolome analysis on an antibody fragment (Fab)-producing *P. pastoris* strain growing in carbon-limited chemostat cultures under different oxygen availability conditions has been performed, in combination with metabolic flux analysis for each metabolic steady state condition. In addition, transcriptional data from previous cultivation series performed under analogous conditions [3] were incorporated in the analysis, allowing to quantify the different contribution of each “omic” level to the regulation of carbon flux through the glycolysis, which, in contrast to *S. cerevisiae*, showed an important transcriptional regulation of this pathway upon adaptation to hypoxia [4].

Generally, changes in metabolic fluxes due to different oxygen availability conditions were the result of altered mRNA levels and post-transcriptional regulation which, in most cases, was in agreement with measured changes in intracellular metabolite levels. In contrast, recombinant protein production was found to cause a much lower impact on *P. pastoris*'s metabolome.

Overall, the results obtained by combining different “omics” analyses provide a metabolic fingerprint of the impact of oxygen availability and recombinant protein secretion, thus improving the physiological knowledge of the biotechnologically relevant yeast *P. pastoris*.

References

Analyzing the early secretory pathway in *Pichia*

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*Pichia pastoris* is unique among budding yeasts in having a secretory pathway organization resembling that seen in more complex eukaryotes. Each *Pichia* cell contains about 3-4 Golgi stacks, each of which is closely associated with a transitional ER (tER) site that produces COPII-coated transport vesicles. By contrast, in *Saccharomyces*, the Golgi consists of individual cisternae that are scattered throughout the cytoplasm, and the tER is correspondingly fragmented. We are interested in elucidating the molecular basis for this striking difference in compartmental organization. In addition, we are taking advantage of the favorable properties of *Pichia* to explore basic questions about the secretory pathway.

Confocal 4D microscopy revealed that *Pichia* tER sites are long-lived structures that form de novo, fuse upon collision, and grow or shrink to attain a steady-state size. This behavior can be explained by a self-organization model. Specifically, we postulate that capture of new tER components is balanced by shrinkage driven by the budding of COPII vesicles. To test this model, we used a genetic screen to identify Sec16 as a key player in tER organization. Sec16 is a large peripheral ER membrane protein that interacts with multiple COPII components. Our results suggest that Sec16 acts as a negative regulator of ER export. According to this view, Sec16 functions primarily to control tER dynamics, rather than to nucleate tER site formation as is generally believed. These data imply that Sec16 defines a new level of regulation for the ER export system.

Protein transport through the secretory pathway has been visualized by live-cell imaging in mammalian cells, but not in budding yeasts. We are attempting to engineer a GFP fusion protein that can be accumulated in the lumen of the yeast ER, and then released in a fluorescent wave for transport to and through the Golgi. This tool will be useful for addressing fundamental questions about whether traffic through the Golgi occurs by cisternal maturation or by a different mechanism.

References


Predicting protein secretion from *Pichia* based on protein stability

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The application of yeasts and filamentous fungi as cell factories for the secreted production of peptides and proteins is commonly used by biopharmaceutical companies and academic researchers. As with all expression systems, the yield of heterologous proteins is usually much lower than desired leading to increased problems and costs downstream. Despite this, most proteins secreted by fungal cell factories are folded correctly and therefore maintain authentic structure and activity. However, the innate quality control mechanisms of the eukaryotic cell, which ensure correct folding of homologous proteins can also target heterologous proteins for degradation. Therefore, it is important to understand stress and homeostasis, how the cell identifies these proteins as foreign and the mechanisms by which they become targeted for either aggregation or degradation. If these factors (bottlenecks) can be identified at the transcriptional, proteome and metabolic levels, it would be a major step towards improving yield and ensuring the authenticity and quality of secreted heterologous proteins. It was the scope of this study to identify bottlenecks associated with heterologous protein secretion in *Pichia pastoris*. Furthermore, we employed a series of folding variants of human lysozyme expressed in *P. pastoris* to test the hypothesis that folded state stabilities of target heterologous proteins determine, at least in part, the secreted yields. The importance of folded state stabilities was confirmed and provides a basis for predicting the secreted yield of a new target heterologous protein. The model was tested by engineering a scFv to improve its folded state stability and we demonstrated that the secreted yield of the engineered scFv was increased, while retaining its functionality. The results of our study also suggest improvements to process design, not only for engineering the producer organism, but also for medium formulation, and the fermentation regime employed.
Reverse Engineering of Protein Secretion by Uncoupling of Cell Cycle Phases From Growth

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The demand for recombinant proteins both for biopharmaceutical and technical applications is rapidly growing, and therefore the need to establish highly productive expression systems is steadily increasing. In yeasts, such as *Pichia pastoris*, protein secretion is a limiting factor of productivity. There is strong evidence that secretion is coupled to the specific growth rate (µ) in yeast, being higher at higher µ. Standard fed batch processes for protein production need to run at low µ, thus limiting the space time yield (STY) of the process. For maximum productivity and product titer, high specific secretion rates at low µ would be desired.

Detailed analysis demonstrated that higher secretion rates correlate to a large fraction of cells in the cell cycle phases G2 and M, which is also supported by literature. Consequently, we designed an improved profile of the correlation of secretion vs. growth by engineering the cell cycle to find more cells in the more productive phases. The cell design target of a higher fraction of cells in G2+M phase was achieved by constitutive overexpression of the cyclin gene CLB2. Together with predictive process modelling, a reverse engineered production strain (secreting an antibody Fab fragment) showed an 18% higher STY and 53% higher product titer. This concept was verified with another secreted protein, human trypsinogen.
Engineering of Protein Folding & Secretion in *Pichia pastoris* –
A systems biology approach

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*Pichia pastoris* is a valuable host for production of heterologous proteins. However, productivities are often limited at the level of protein folding and secretion. To accelerate the development of production strains it is crucial to understand the molecular physiology of the host, and the specific limitations that the product may exert on expression. Furthermore, the complexity of protein synthesis and secretion renders protein production much more challenging to be addressed by methods of systems biology compared to metabolite production.

In order to establish a genome scale approach to analysis and engineering of *P. pastoris*, we have sequenced the genome, developed a post genomics platform including a genome scale metabolic model and employed them for the analysis of cellular reactions to intrinsic stress and extrinsic conditions of bioprocesses. Combined transcriptomic, proteomic and metabolomic data will be presented which indicate why environmental conditions like temperature, oxygen supply or osmolarity have a strong impact on heterologous protein production.

Additionally we have applied high throughput methods such as DNA microarrays and cell sorting for the direct screening of genes encoding factors which support protein secretion. This enabled the rapid selection of factors enhancing protein productivity several fold. Moreover, the results provide a pattern of subcellular processes which represent bottlenecks of heterologous protein secretion, thus closing the systems biology cycle by opening further possibilities for strain engineering.
Pichia pastoris genome 2.0: higher accuracy, improved annotation and a workflow to find point mutations through resequencing


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We determined and annotated the genome sequence of the methylotrophic yeast Pichia pastoris strain GS115 in 2009 [1]. This widely used histidine auxotrophic GS115 strain was derived more than two decades ago by random chemical mutagenesis from the parental strain NRRL Y-11430/CBS7435. The NRRL-Y11430/CBS7435 genome has now also been sequenced, using dual sequencing on the Roche/454 and Illumina platforms (ref. [2]; Chappell, T. and Cregg, J.M., unpublished data). The GS115 reference sequence genome assembly was solely based on the 454 platform, with an assembly error rate better 1/30,000, i.e. containing ~300 errors, mostly in homopolymer regions. We have now also sequenced the GS115 genome on the Illumina platform and we have used all available genomic data from our joint laboratories to obtain an updated reference sequence, which allowed for a higher-precision gene annotation. RNAseq and proteomics data (Oliver, S., unpublished data) were available to validate the gene models of ORFs with reduced confidence scores from the gene prediction algorithms. Gene expression micro-arrays (Agilent platform) are now also available for Pichia [3] and the probe sequences were mapped to the reference sequence. The entire updated Pichia pastoris genomics resource is publicly available online [4,5]. Random mutagenesis and selection is a very successful technique in industrial microbial strain engineering but it has been difficult to pinpoint the phenotype-causative mutations. This hampers the rational re-use of such mutations in novel projects. From the comparative genome analysis of the parental strain NRRL Y-11430/CBS7435 and its NTG-mutagenized GS115 derivative, we show that it is possible to reliably define point mutations when sequencing both parent and daughter strain using both Roche/454 and Illumina platforms. Both sequencing platforms have a systematic sequencing error structure which is different and integration of the reads of both platforms allows to cross-correct such sequencing error. With a number of exceptions, the Pichia genome is remarkably free of repetitive sequences and lends itself well to this kind of resequencing effort. With
the rapidly dropping cost of the required sequencing datasets, this workflow becomes economical and should open up new avenues in *Pichia* strain engineering.

References
Get product quality under control!
Factors affecting the variability of heterologous proteins during production processes with recombinant *P. pastoris*

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Safety concerns, decreased functionality, and high product purification costs together with lower product yields are currently prompting an investigation of factors which cause variability in proteins during their biotechnological production. The targets of molecular strain design have evolved beyond the initial focusing on high productivities and titres. Recent advances in the engineering of *Pichia pastoris* strains to efficiently conduct specific types of post-translational modifications (PTMs) promise highly homogenous as well as novel PTMs. Despite these forward-looking opportunities in strain design, conditions in the bioreactor (under which the biomass is grown and the product built) are crucial to avoiding variations in molecular structure and/or function of the target recombinant protein.

Efficient production processes with *P. pastoris* typically require high cell densities, which are feasible in fedbatch operation. During a fedbatch process, controlled substrate addition enables a culture to be grown at a predefined specific growth rate and the associated cellular processes/fluxes also to be controlled. However, while the carbon/energy substrate is added, the culture volume increases and the biomass and/or product formed accumulate in the bioreactor with all the impurities (various product variants) which have formed since the beginning of the process. The experimental programme outlined seeks deeper understanding of the impact of controlling culture (environmental) conditions in biotechnological production processes on the variability of heterologous proteins produced with *Pichia pastoris*. For two models, an enzyme and a therapeutic protein, variations in the specific enzyme activity and the occupancy of the N-glycosylation sites as well as N-glycan homogeneity were found to be related to both the specific growth rate and various carbon/energy substrates.

This knowledge can be used for the rational design and optimisation of manufacturing processes to simultaneously achieve maximum productivity and the highest possible (homogenous) product quality. It also provides valuable feedback from which molecular strain construction can be improved. Thus, product variability, which might be introduced by an inappropriate cultivation strategy, can be eliminated and controlled by a suitable one, and the emerging generation of recombinant strains can be used to their full potential.
Improving the biophysical properties of full-length IgG – inspired by *Pichia pastoris*

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Antibodies and their derivatives have found a broad range of applications, from basic research to medical therapy. As part of their functions relies on binding to Fc receptors and therefore on the N-linked glycosylation in their CH2 domain, currently all approved monoclonal antibodies are produced in mammalian cell lines. Recently, however, *Pichia pastoris* has also been engineered to introduce complex, human-like glycosylation [1, 2] - thus expanding the range of possible production hosts to this yeast system. Here, we compare the influence of the choice of production system (mammalian HEK 293 cells vs. *P. pastoris*) on the biophysical properties of the expressed antibodies.

In our analysis of immunoglobulin G molecules (IgGs) of identical amino acid sequence but produced in either of these systems, dramatic differences in their aggregation susceptibilities were encountered. The antibodies produced in *P. pastoris* were much more resistant to aggregation under many conditions, a phenomenon found to be mainly caused by two factors. First, the mannose-rich glycan of the IgG from *P. pastoris*, while slightly thermally destabilizing the IgG, strongly inhibited its aggregation susceptibility, compared to the complex mammalian glycan. Second, on the *P. pastoris* produced IgGs, amino acids belonging to the α-factor pre-pro sequence were left at the N-termini of both chains due to imperfect processing by the enzyme dipeptidyl aminopeptidase A (encoded by STE 13). These additional residues proved to considerably increase the temperature of the onset of aggregation and reduced the aggregate formation after extended incubation at elevated temperatures. The attachment of these residues to mammalian IgGs confirmed their beneficial effect on the aggregation resistance – indicating the transferability of this finding. Secretion of correctly processed IgGs in the yeast system became also possible after systematic engineering of the precursor proteins and the processing site. This allowed the definition of optimal sequence composition for either aggregation-resistant or correctly processed IgGs, respectively.

Taken together, the presented results will be useful for the successful production of full-length IgGs in *Pichia pastoris*, give indications on how to engineer aggregation-resistant IgGs and shed new light on potential biophysical effects of tag sequences in general.

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Strategy Development for Therapeutic Protein Production by *Pichia pastoris*

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Strategy development for the therapeutic protein production by *P.pastoris* for the two therapeutic recombinant proteins, i.e., a glycoprotein hormone, erythropoietin (EPO), and a non-glycosylated protein, human growth hormone (hGH), will be presented. EPO chiefly regulates the production of red blood cells, and is produced primarily by the kidney in the adult and by the liver during fetal life; whereas, hGH benefits the treatment of hypopituitary dwarfism, growth hormone deficiency, Turner’s syndrome, chronic renal failure and human immune deficiency virus (HIV) syndrome.

An industrial microorganism, as the micro-bioreactor within the bioreactor, function with strong interactions within its micro-environment in an aerobic bioprocess that should be properly formed by the design of the medium with the oxygen transfer and pH conditions [1], within a bioreactor operation strategy. The influence of feeding strategy and pH operation condition on the two therapeutic proteins, one of which is glycosylated (rEPO), and the other one (rhGH) is non-glycosylated by *P.pastoris* showed diverse effects. For rEPO production, for the first three phases of the bioprocess, glycerol batch (GB), glycerol fed-batch (GFB) and methanol transition (MT), the growth profiles were almost the same for pH≥5.0; however, at pH<5.0 the growth rate was lower. The highest cell concentration was obtained as 81.4 g L⁻¹ where GB and GFB were operated at pH=5.0 and MT and protein production phase (PP: sorbitol was added to the medium in batch-wise and r-protein production was induced by the addition of methanol in fed-batch mode, where the pre-fixed growth rate was taken as, μ = 0.03 h⁻¹) were operated at pH=4.5, resulting in the highest rhHuEPO concentration as 0.16 g L⁻¹ [2,3]. On the other hand, with the same carbon source feeding strategy, for rhGH production the most favourable pH was 5.0; where AOX1 expression level showed a similar trend to AOX activity profiles, in parallel to AOX1 expression and hGH expression levels; They increased until t = 15 h, and the rhGH concentration was obtained. 0.27 g L⁻¹ [4], being 1.7-fold higher than that of rEPO. With the development of the feeding strategy based on sorbitol-methanol carbon sources the highest rhGH production was obtained as 0.6 g L⁻¹ at t = 48 h, where the cell concentration was 105 g L⁻¹.

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Development of an optimized sequential production and purification process for recombinant proteins with *Pichia pastoris* in a fully automated integrated pilot plant

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A fully automated integrated bioplant was developed for the production of recombinant proteins like an industrial enzyme lipase CALB or a potential Malaria vaccine D1M1his with *Pichia pastoris*. The pilot plant combines upstream and downstream operations to produce and purify the target proteins in a long term sequential and parallel process.

Cultivation takes place in two bioreactors. In a cyclic cell breeding process fresh cells are cultivated daily on glycerol with a final methanol pre-induction. After a transfer to the expression reactor the production of the target protein last another 24 hours.

The downstream part consists of a disc clarifier, cross flow filtration units and an ÄKTApurifier 100 for a final purification step. The possibility to extract intracellular proteins is given by a high pressure cell disruption unit.

The downstream of CALB was performed with a separation of cells, a microfiltration of cell debris and the purification by hydrophobic interaction chromatography (HIC). The pharmaceutical protein requires an additional ultrafiltration step to perform a following immobilized metal affinity chromatography (IMAC).

The downstream process last less than 24 hours, so the whole production process can be performed in three days but the sequential and parallel processing enables a harvest of one batch per day.

DoE-Design of Experiments was used for optimizing all upstream and downstream operations. For protein expression a common DoE-strategy was applied successfully. As a result the productivity of enzyme production was increased fivefold.

In the microfiltration step the recovery of target protein was maximized by DoE with the variation of feed, retentate and permeate pressure and the pH value in the inlet. In the final protein purification also an optimization of the target protein recovery was carried out by varying the pH value and the retention times of binding and elution.

The presentation includes the description of assembling and automation of a complete scale down production plant. Strategies of sequential time shifted parallel processes as well as optimization of each unit operation is explained in detail.
Optimization of a sequential pharmaceutical protein production process with in-situ product removal (ISPR)


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The continuous production process for recombinant proteins is the goal for an industrial production in the near future as it was presented at the 1st European Congress in Applied Biotechnology in Berlin in September 2011 [1]. These days the operation mode for a recombinant protein production is usually batch wise. In this article the cultivation and product capture process was carried out in a sequential manner, which means that the cell growth, protein expression and protein capture run iteratively in cycles for several times. With this sequential operation mode an optimization by design of experiments (DoE) could be realized timesaving. Additionally the sequential process can be easily turned into a continuous one. A continuous outflow of target protein from the last chromatographic polishing step can be realized by applying cyclic column load and elution by use of two or more columns.

Here a malaria vaccine candidate was expressed and secreted by P. pastoris. The in-situ product removal (ISPR) was realized by Expanded Bed Adsorption (EBA) chromatography. This article initially will present the implementation of a sequential cultivation process with integrated product removal by EBA. Afterwards the focus will be on the demonstration of the optimization with DoE in each unit operation, cultivation, protein capture and purification, respectively.

The cultivation of P. pastoris is a multi phase and two-substrate process. Glycerol is used for cell growth and methanol for the induction of recombinant protein expression. A sequential process strategy has to guarantee the same cell status at the beginning of a new cycle, especially in case of DoE experiments. The methanol memory after switching back to glycerol can be erased. That was proved by cell internal alcohol oxidase activity determination.

DoE was applied to the cultivation process, where the variables temperature, pH and methanol concentration were optimized. The protein capture via EBA was pre-optimized in a down-scale FPLC plant and finalized as an integrated unit-operation at the cultivation process. Here the variables flow rate, pH, conductivity, imidazole and Tween® 20 concentrations were selected for screening and optimization. Pooled fractions from the EBA were purified by gel permeation chromatography. This last step was optimized in terms of flow rate and application volume.

All unit operations of the process could be optimized in productivity (protein amount) and product quality (less degradation products). The process and optimization strategy can be easily transferred to other target proteins. The sequential operation mode can be accomplished to a continuous product flow quite simple by above mentioned cyclic use of several chromatography columns.

References
A Systems and Synthetic Biology framework for pathway level culture media engineering: application to Pichia pastoris


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Culture media (CM) formulations contain hundreds of ingredients in water solutions that may be involved in complex interactions in the same or competing pathways within the cell. The standard methodology for determining the optimal composition of CM is based on intensive experimentation supported by statistical design-of-experiments (DoE). However, due to its empirical nature, DoE based design methods are time consuming and costly procedures when applied to many medium factors with potential interactions, as the number of screening experiments increases geometrically with the number of medium factors. Moreover, because the function of medium factors is most of the times unknown, adjustment of optimal medium composition to different strains expressing different products is essentially a black-box procedure.

In this project we have developed a Systems Biology framework to streamline culture media engineering. A basic premise was to migrate from an empirical to a mechanistic or hybrid mechanistic/empirical CM design approach. A framework consisting in the execution of an array of cell cultures, endpoint exometabolomic assays and bioinformatics algorithm were brought together into a platform for CM engineering called cell functional enviromics [1]. Cell functional enviromics consists of a large-scale reverse engineering approach that reconstructs cellular function on the basis of measured dynamic exometabolome data. To support this concept, a computational algorithm was developed, called “envirome-guided projection to latent pathways” [1]. This method yields envirome-wide functional enviromics maps, with rows representing medium factors, columns representing elementary (orthogonal) cellular functions and color intensity values, the strength of up-/down-regulation of cellular functions by medium factors.

This framework was applied to the engineering of chemically defined CM for the yeast Pichia pastoris to improve heterologous protein expression. An array of shake flask experiments were performed and used to build a P. pastoris functional enviromics map. Then, optimised medium formulations were calculated targeting predefined heterologous protein expression improvements. X33 and GS115 strains expressing three different proteins (scFv, HAS and DNAase) were employed in this study. Several shake flask experiments were carried out according to the optimized CM formulations. The results were benchmarked against the BSM recipe by Invitrogen. In general, our designs had adverse effect on cell growth rate but a very positive impact on the specific production of heterologous proteins with over 100% increase in productivity. Furthermore, 50 L pilot reactor experiments confirm the shake flasks results with a productivity increase and maximum titer increase of approximately twofold in relation to the control BSM recipe.

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Regulation of catabolite repression of AOX1 promoter by Mig in *Pichia pastoris*


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In this report, two catabolite repressor genes (*PpMIG1* and *PpMIG2*) were cloned in *Pichia pastoris*. Both *PpMIG* genes encode the proteins homologous to the DNA-binding repressor Mig1 from *S. cerevisiae* (*ScMig1*), which is a C2H2 zinc finger protein and can bind to the promoters of many genes repressed by glucose. *PpMig1* and *PpMig2* locate in nucleus but not cytoplasm in glycerol medium, which is different from *ScMig1*. When cells are transferred to methanol, *PpMig1* and *PpMig2* are exported from the nucleus and then locate in the cytoplasm.

In addition, *PpMig1* plays an important role in catabolite repression of alcohol oxidase I gene (*AOX1*). Notably, we firstly found that *PpMIG1* deletion or *PpMIG1* and *PpMIG2* double-deletion, but not *PpMIG2* deletion, leads to the induced expression of *AOX1* gene in response to glycerol, but not glucose. Heterologous proteins derived by *AOX1* promoter could also be induced by glycerol in *mig1mig2* mutant.

Genomewide RNA-Seq analysis of *mig1mig2* mutant versus wild-type strain grown in glycerol revealed that numerous genes changed their expression in the absence of *PpMig1* and *PpMig2*. Nearly 7% (358 genes) of the approximately 5277 annotated *P. pastoris* genes were significantly up-regulated with at least a two-fold differential expression in mutant. Highest up-regulation (> 30-fold) was observed for the genes encoding the AOX1, a key enzyme of the methanol dissimilation pathway. Up-regulated genes also included genes encoding other enzymes of methanol metabolism. Approximately 23% (1197 genes) of the genes were down-regulated. These included mitochondrial translation genes as well as genes involved in mitochondrial respiratory chain complex assembly and mitochondrial electron transport.
Doing the splits – yielding g/L levels with methanol-driven and methanol-free production from VTU´s AOX1 promoter library

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Ease of genetic manipulation, rapid scalability and high-level production of secreted recombinant proteins are some of the reasons for the evolution of the methylotrophic yeast *Pichia pastoris* to an industrially relevant host organism. One of the features leveraging *Pichia*´s importance, its strong AOX1 promoter which is used in the majority of cases for protein production, at the same time represents a potential downside of this yeast: mandatory usage of methanol for heterologous gene expression. While some manufacturers are well-equipped for this toxic and explosive reagent in large scale, others do not want to handle it, or can´t. Still, because current constitutive promoters do not keep up with AOX1 promoter-driven production levels, methanol induction is the process of choice.

VTU Technology´s 1st generation AOX1 promoter library focused on ever higher production levels of recombinant proteins using methanol as inductor, reaching titers as high as 20 g/L of secreted protein. Now, 2nd generation AOX1 promoter variants were generated that bear a higher threshold concentration of glycerol or glucose to fall into a “derepression status” and promote efficient transcription of the target gene(s) upon limited glycerol/glucose feed. Although in most cases protein production levels are below a methanol-driven approach, in general 30-70% as compared to the best methanol-induced process with 1st generation promoters are reached for many target proteins, while process times can be reduced significantly.

Hence, a high-level protein production system with *Pichia pastoris* was generated that is able to do the split between dual expression approaches for recombinant proteins, methanol-induced as well as methanol-free production, depending on the particular requirements. In combination with VTU Technology´s approved microscale cultivation and high throughput screening system handling >20.000 clones per week, a powerful eukaryotic competitor to bacterial organisms was developed.
Purification of AOX1 promoter-binding proteins from Pichia pastoris

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The methylotrophic yeast Pichia pastoris is used as a host system to produce recombinant proteins for a variety of applications, from food enzymes to pharmaceuticals. It is also used as a model organism to study the eukaryotic gene expression and peroxisome biogenesis. P. pastoris has many advantages over prokaryotic systems, including the ability to perform many eukaryotic post-transcriptional modifications, and the capability of this yeast to produce heterologous proteins at high levels extracellularly. Although the AOX1 promoter is one of the most extensively utilized promoters for recombinant expression in eukaryotic systems, methanol induction mechanism is not understood well. Only, Methanol expression regulatory protein 1 (Mrx1p) was identified as the regulator of the methanol utilization pathway gene.

In order to understand how regulation is imposed on AOX1 promoter, we used systematic deletions to identify regions of the promoter element involved in regulation, and DNA-protein interactions were investigated. Using the sequential deletion analysis, the AOX1 gene was divided into subfragments. The Escherichia coli lacZ gene was used as a reporter to measure promoter activity. Deletion analysis revealed that each fragment had a significant effect on the promoter activity. A protein complex consist of three subunits that bind a fragment of the AOX1 promoter was purified. In this presentation, the AOX1 promoter deletion analysis and purification of DNA binding proteins will be discussed.
Playing with the methanol utilization pathway in *Pichia pastoris*


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**Motivation**

Alcohol oxidase is the key enzyme for methanol utilization (Mut) in the methylotrophic yeast *Pichia pastoris*. However, also other enzymes along the Mut pathway play a crucial role especially when it comes to the metabolism of produced formaldehyde directing it either to the assimilation or dissimilation pathway. Overexpressing either of these enzymes should significantly affect methanol metabolism and thus probably also protein production of recombinant *P. pastoris* strains.

**Results**

We recently developed a novel, generally applicable approach based on fast and easy to do batch cultivations with methanol pulses which enables a more rapid determination of strain specific parameters and thus allows a reliable and fast strain characterization and speeds up process development [1]. We applied this strategy (Figure 1) to compare a Mut$^+$ strain and a Mut$^-$ strain expressing the recombinant model-enzyme horseradish peroxidase. When the specific substrate uptake rate ($q_s$) and the specific productivity ($q_p$) were put in relation, the Mut$^-$ strain turned out to be much more efficient than the Mut$^+$ strain. Consequently we overexpressed the MUT pathway enzymes dihydroxyacetone synthase (DAS1), formaldehyde dehydrogenase (FLD1) and transketolase (TKL1) in recombinant *P. pastoris* Mut$^-$ strains expressing either *Candida antarctica* lipase B (CalB) or horseradish peroxidase (HRP). Unexpectedly, overexpression of the MUT pathway enzymes did not have any effect on biomass and off-gas yields - the energy balance of the cells remained unimpaired. However, dramatic changes in $q_s$ and $q_p$ were observed (Figure 2). Strains overexpressing DAS1 could produce 3 times more recombinant enzyme with respect to the consumed substrate methanol than the benchmark strain. Since the conversion from substrate into product was much more efficient for this strain, it could be interesting for industrial large-scale production processes, also in terms of a reduced risk management.
Reference

Higher titres with less methanol – cultivation of *Pichia pastoris* Mut⁺ with mixed substrates

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Methanol, which use is necessary in processes producing recombinant proteins cloned under control of AOX1 promoter and which usually serves as both inducer of AOX1 to express foreign gene and source of carbon and energy to support *Pichia pastoris* growth, is not an ideal substrate from technological point of view. Challenges such as necessity for high aeration rate and intensive bioreactor cooling due to a high oxygen demand associated with methanol metabolism, a need for an accurate methanol dosing control system to prevent its accumulation in bioreactor and methanol fire hazard must often be solved. Some of these problems can be reduced by replacing a part of methanol with other substrate, which allows achieve both high biomass yield and foreign protein productivity, i.e. to use a “mixed feeding strategy”.

Glucose, glycerol and sorbitol were tested as alternatives for a part of methanol in processes producing porcine trypsinogen with *Pichia pastoris* Mut⁺ strain. The carbon-limited chemostat cultures performed at low dilution rate of 0.03 h⁻¹ (selected in previous experiments as an optimum for extracellular trypsinogen production) were applied to compare the growth of *P. pastoris* and trypsinogen production on above mentioned substrates or their mixtures with methanol (1:1). In carbon/energy limited cultures both methanol and “complementary” substrate were consumed simultaneously and enabled to achieve both higher biomass yields and trypsinogen titres compared to sole methanol. AOX1 was expressed in all “mixed experiments” and in cultures with sole sorbitol; no alcoholoxidase activity was detected in experiments performed with sole glucose and glycerol.

A fed-batch protocol consisting of 1) high exponential feeding of “repressive” substrate to keep the growth rate close to 0.2 h⁻¹ to achieve high concentration of biomass and 2) low exponential feeding of sole methanol or methanol plus complementary substrate (1:1) to express foreign gene and keep the specific growth rate at 0.03 h⁻¹ was finally designed. A yield exceeding 1 g l⁻¹ of porcine trypsinogen was repeatedly achieved with mixed substrates.
Application of fed-batch screening at microscale and scale-up to laboratory fermenter with a lipase expressing *Pichia pastoris* strain


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Rapid development of optimized processes for recombinant protein production is essential for achieving process productivity levels that will support commercial production of biologics using microbial expression systems such as *Pichia pastoris*. The development of small/micro-scale multiplexed systems mimicking conventional bioreactor systems behavior is therefore key for fast bioprocess development and scale up. These systems can be used to select an optimized strain, growth media, feed strategy and other bioreactor parameters. Once determined, these optimized conditions can then be tested in bench scale bioreactors to demonstrate process reproducibility and scalability. The m2p-labs microbioreactor platform, BioLector, has already been tested successfully for clone screening, media optimization and scale up for batch processes. In this study, we have investigated the usefulness of this system for strain screening and development of recombinant protein production processes based on the classical methanol-regulated *P. pastoris* expression system. The new Feed-In-Time (FIT) fed-batch media are introduced with an exemplary study of Lipase expressing *Pichia pastoris* clones. The FIT fed-batch-media possess the capability to release glucose from inert polysaccharides by enzymatic hydrolysis directly in the medium and therefore, are ideally suited for small scale applications. A typical workflow in industrial biotechnology was imitated with PAOX1 driven expression of *Rhizopus oryzae* lipase (RoL) in *Pichia pastoris* Mut+ phenotype as example. Both initial clone screening and development of cultivation strategy were exclusively conducted in the micro-scale using 48 well Flowerplates in combination with the BioLector technology for online monitoring of the most relevant cultivation parameters (biomass, pH and DO). Therefore, the first steps of a glucose/MeOH based bioprocess development could be carried out in a time-efficient manner and led to lots of valuable data and thus, gave comprehensive understanding of the bioprocess to be developed. Namely, the impact of batch vs. fed-batch fermentation and different induction and feeding strategies to obtain high cell densities (OD<sub>600</sub> ≈ 300) and lipase activities (≈ 25 kU/L) were investigated yielding a cost-effective and high-producing bioprocess to be transferred to classical stirred tank fermentations. Furthermore, the best cultivation conditions determined in 48 well Flowerplates using mixed substrate (glucose + MeOH) feeding strategies were also implemented in a 5-liter bioreactor. Two different methanol feeding rates strategies were tested with the aim to validate the scalability of the microscale fed-batch strategies: i) maintaining a constant methanol concentration and, ii) a methanol pulse addition. Lab-scale cultivations also allowed validating the selection of an improved growth medium, as well as the performance of the best clone identified from Flowerplate cultures. Overall, the results obtained in lab-scale conventional bioreactors demonstrate the reliability/suitability, reproducibility and scalability of the Flowerplate microscale cultivation system for the *P. pastoris* system.
Making synthetic proteins in *Pichia pastoris*

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Synthetic proteins can be produced *in vivo* by using non-canonical amino acids as building blocks. Though not encoded by the genetic code, these analogs of the canonical amino acids participate in protein translation under tightly controlled conditions. In fact, a suitable auxotrophic host is supplemented with the non-canonical amino acid during the expression of the target protein. The analog then occurs at all positions specified by the codon for the related canonical amino acid. Most of the non-canonical amino acids carry unusual side chains. Their translation into a target protein sequence can provoke structural, chemical, or functional modifications normally not found in nature.

While the production of synthetic proteins has become routine in *E. coli*, the suitability of *Pichia pastoris* for this application remains largely unexplored. *Pichia pastoris* is a popular eukaryotic expression host for industrially relevant proteins, particularly if post-translational modifications or secretion into the medium are desired.

In order to explore *Pichia pastoris* as a host for synthetic protein production, we expressed the lipase B from *Candida antarctica* (CalB) in appropriately auxotrophic strains supplemented with different amino acid analogs. The CalB variant containing norleucine, a hydrophobic derivative of methionine, exhibited improved enzyme activity in comparison to the unsubstituted parent protein. This finding parallels a similar observation with a bacterial lipase. It is the first indication that the incorporation of a specific amino acid analog into different members of the same protein family elicits comparable effects. CalB was expressed with a number of other methionine analogs, however, they either did not affect lipase activity, impaired it, or eliminated it entirely.

In a parallel experiment, we exchanged the tyrosine, phenylalanine, and tryptophan residues of CalB by their fluorinated analogs. The CalB variants containing fluorophenylalanine or fluorotyrosine showed improved shelf life because they were noticeably more active than the parent lipase after several months at 4 °C. The analog incorporation did neither interfere with the glycosylation of CalB nor with its efficient secretion into the medium.

Our results clearly demonstrate the suitability of *Pichia pastoris* as an expression host for synthetic proteins containing non-canonical amino acids at multiple positions.

A biotechnologically relevant protein, CalB, was furnished with desirable traits such as improved catalytic activity and prolonged shelf life by simply substituting amino acids by their non-canonical analogs. Elaborate DNA mutagenesis steps were not necessary.

Nevertheless, to establish *Pichia pastoris* as a production host of synthetic proteins, the technique awaits validation with different kinds of proteins routinely expressed in this yeast.

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**Pichia pastoris** as Whole-Cell Biocatalyst for (±)-Nootkatone Production

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*Pichia pastoris* was used for heterologous expression of *H. muticus* premnaspirodiene oxygenase (HPO; CYP71D55) in combination with a cytochrome P450 reductase (CPR) from *A. thaliana*. The plant P450 monoxygenase HPO has been shown previously to convert the sesquiterpene (±)-valencene, which is a natural aroma compound of citrus fruits, yielding the highly sought-after grapefruit flavor (±)-nootkatone in *S. cerevisiae* [1]. Co-expression of HPO and CPR in *P. pastoris* was monitored by CO-difference spectra measurements and activity assays, respectively. Whole-cell biotransformation of (±)-valencene resulted in the production of trans-nootkatol, which was subsequently oxidized to (±)-nootkatone by an intrinsic *P. pastoris* activity. Cellular uptake of most hydrophobic (±)-valencene was found to be a bottleneck in (±)-nootkatone formation, which was overcome by intracellular (±)-valencene synthesis. Oxidation of trans-nootkatol was enhanced by co-expressing alcohol dehydrogenases. Thus, the yeast *P. pastoris* is a competitive whole-cell system for high-level production of (±)-nootkatone.

Reference

Commercializing the Best Enzymes from Nature

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More than three and a half billion years of natural evolution has led to the tremendous diversity of microbial life on our planet. At Verenium, we have developed a suite of proprietary technologies to unlock the secrets of untapped microbial genomes in order to develop new and versatile enzymes. Realizing value from biodiversity is the foundation of our sustainable business and also provides a compelling argument for the preservation of the world's natural habitats. In this presentation, we will describe some of our recent work on the discovery, evolution, and commercial application of high-performance industrial enzyme products. Moreover, we will discuss the central role of heterologous expression technologies in creating robust scalable bioprocesses for efficient enzyme production, highlighting the utility of Pichia.
Nanobodies from bench to bedside, with a big help of Pichia

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Ablynx is a biopharmaceutical company engaged in the discovery and development of Nanobodies®, a novel class of therapeutic proteins based on single-domain antibody fragments, for a range of serious and life-threatening human diseases, including inflammation, haematology, oncology and pulmonary disease. Today, the company has over 25 projects in the pipeline and six Nanobodies in clinical development. We demonstrated the high expressability of Nanobodies in mammalian (CHO cells) and microbial systems (E. Coli). However, an optimized Pichia pastoris expression platform resulted in sharp development timelines and allowed efficient identification of lead candidates with excellent development properties. Insight will be given into the setup of our generic Pichia expression platform and its optimization and use in discovery and development of therapeutic Nanobodies.
High throughput methods for *Pichia pastoris* pave the way for rapid process development

Case study: Production of novel therapeutic proteins fused to human serum albumin as half life extension


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The methylotrophic *P. pastoris* has become the predominant yeast for recombinant protein production. Its ability to efficiently secrete target proteins to the culture supernatant enables the development of cost and time saving processes for protein purification. Furthermore, genetic tools like strong and tightly regulated promoters as well as solid fermentation processes were developed that allow for commercial, high titer production of a wide range of heterologous proteins. Here, a case study for the production of a therapeutic protein using *P. pastoris* is presented. Since human serum albumin (HSA) is known to increase the duration of stay of drugs within the blood plasma, the strategy of HSA-fusion was chosen as half life extension for the evaluated therapeutic protein.

Employing the technique of pool transformation, more than 1000 different *P. pastoris* clones were generated each carrying the HSA-fusion gene under control of one of VTU’s proprietary, methanol-inducible promoters. These were screened in a high throughput manner for target protein production. After initial screenings in micro well scale, high producing clones were chosen for bioreactor fermentations at lab scale. In these initial fermentations, high product titers in g/L range were reached. Automated high-throughput downstream development subsequently allowed for the establishment of a lab scale purification process within little more than four weeks of development time with excellent overall yields.
Analyses of a Monoclonal Antibody Produced by *Pichia pastoris*

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In recent years, development of biologics like monoclonal antibodies (mAbs) has expanded, and, more inexpensive production system is required. We have developed a recombinant expression system producing a therapeutically important mAb, trastuzumab against the HER2, by *Pichia pastoris* GS115. Codon-optimized synthetic genes encoding the heavy and light chains of trastuzumab were introduced under the *AOX1* promoter independently. Hen egg white lysozyme signal sequence was used for secretion of the heavy and light chains to *Pichia* culture medium. A fragment of an upper region of the *AOX1* promoter was introduced to the expression vector to enable integration of the heavy and light chain genes onto the host chromosome by homologous recombination. Transformants having the DNA copies of the heavy and light chains were screened by colony PCR. Positive clones were cultured and culture supernatants were purified by Protein A column chromatography. *Pichia*-derived trastuzumab was detected by a Western blot under reduced condition using an anti-human IgG1-Fc antibody and an anti-human Lkappa antibody. Coomassie blue stained SDS-PAGE analysis of non-reduced sample demonstrated that *Pichia*-derived trastuzumab had correct heterotetrameric folding. In addition, the results of HER2-binding activities and its glycan structures of *Pichia*-derived trastuzumab will also be presented.
Production of a malaria transmission blocking vaccine candidate Pfs25 in Pichia pastoris for human clinical studies

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Approximately one-half of the world’s population lives in areas exposed to the malaria parasite Plasmodium falciparum resulting in an estimated million deaths annually, 85% of which occur in children under 5 in sub-Saharan Africa. The financial cost of mosquito-transmitted malaria cripples economic development and contributes to the economic disadvantage of many countries. The development of a transmission blocking P. falciparum malaria vaccine is considered critical for future control measures of elimination and eradication. To this end, a malaria transmission blocking vaccine against an ookinete protein, identified as Pfs25 is being pursued which targets the malaria parasite as it reproduces in the mosquito’s gut. Pfs25 contains 4 epidermal growth factor-like domains comprised of a total of 11 disulfide bonds. Human antibodies generated against an experimental Pichia pastoris (Pp) produced Pfs25(H) vaccine when taken up by the mosquito in a standard membrane feeding assay inhibited parasite development and subsequently blocked mosquito infectivity. In order to proceed beyond initial phase I and II testing, a second generation production clone has been produced in order to remove 14 heterologous amino acids including a HIS affinity tag. The second generation PpPfs25(M) production clone was generated using a synthetic Pfs25(M) gene and a P. pastoris protein disulfide isomerase co-expression vector under an AOX1 promoter such that the secreted PpPfs25(M) protein contained no heterologous amino-acids. Fermentation development evaluating induction temperature, pH and methanol feed rates in defined media using a Box-Benken design of experiment model (total of approximately 35 fermentation runs) in 5 liter fermenters yielded product at over 1 gram/liter supernatant of PpPfs25(M) material by an analytical ion-exchange-HPLC method. A robust fermentation process was established and performed at pilot-scale in a 60L working volume fermentation and yields were greater than 1 gram/liter supernatant. PpPfs25(M) has been purified and characterized, and available for bridging studies.
New strategies for induction and screening of high expressing clones

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Pichia pastoris has become one of the most developed expression platforms for the production of both, industrial enzymes and biopharmaceuticals. As stable, autonomously replicating vector systems are not readily available, integration into the genome by homologous or ectopic integration is state of the art for the construction of expression clones.

It is well documented, that besides the use of proper expression signals such as promoters, terminators, signal sequences etc., which primarily drive expression, the final genomic setup of expression clones strongly influences expression and/or secretion levels. One important factor is the number of integrated copies but the general genomic situation such as location of integration and possible rearrangements in the course of the integration process can strongly influence the overall yield of heterologously expressed proteins. As a consequence, the clonal variation of primary transformants can be high and screening larger numbers of clones for high expression capacity provides a useful strategy for expression strain development. Most employed are systems based on screening for increased resistance levels mediated by used antibiotic resistance makers (e.g. Zeocin, Geneticin).

This study reports on the one hand on the identification of a specific genomic setup in a high expression production strain established for industrial production of the plant-derived enzyme hydroxynitrile lyase [1].

Based on the knowledge of this specific setup, it was possible to construct expression vectors reflecting this setup. Furthermore, new concepts for screening high expression clones are reported.

References

Characterization of recombinant protein production in *Pichia pastoris* under control of the novel *THI11* promoter and design of a tailor-made production process

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The methylotrophic yeast *Pichia pastoris* is widely used for production of recombinant proteins. However, expansion of possible applications and improvement of the powerful production platform requires new expression strategies and alternative production processes. Stadlmayr and co-workers [1] identified a number of novel promoters for recombinant protein expression in *P. pastoris*. Thereof the regulatory region of *THI11*, a gene involved in thiamine biosynthesis, has been studied in more detail in this work due to its possible adjustability by thiamine. An X-33 strain, expressing recombinant human serum albumin (HSA) under the control of the *THI11* promoter, has been used to characterize the production kinetics.

Chemostat cultivations under non-repressing conditions and over a wide range of the specific growth rate $\mu$ were performed to evaluate the dependency of the specific production rate $q_P$ on $\mu$, as well as the full potential on the promoter. Media supplemented with different amounts of thiamine were used in batch cultivations in order to gain more knowledge about repression and de-repression kinetics of *THI11* promoter-driven gene expression. Analysis of transcript levels of the native *THI11* and heterologous HSA genes in addition to the determination of intracellular and extracellular thiamine concentrations (HPLC with fluorescence detection) complemented the investigation of the recombinant protein production.

It was found that the *THI11* promoter reveals a constitutive nature under non-repressing conditions, whereas the specific production rate $q_P$ was enhanced at high specific growth rates. Results of the batch fermentations proved the thiamine-sensitivity of the *THI11* promoter. Product formation can be repressed for a desired period of time by using medium supplemented with a certain amount of exogenous thiamine. Based on these results a tailor-made process strategy was designed and experimentally verified. Furthermore we will present a comparison of the productivity of the novel *THI11* promoter and the well known *GAP* promoter in fed batch cultivations.

All the obtained results lead to the conclusion that the novel *THI11* promoter is a suitable candidate for controlling recombinant protein production in *P. pastoris* and can become a valuable tool for further improvement of this widely-used production platform.

References
Production of recombinant proteins under oxygen limited process conditions: scFv production, a case study

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Strong inducible AOX1 promoter, widely used in *P. pastoris* for fast, inexpensive production, is typically induced by methanol. The high oxygen demand of methanol metabolism makes oxygen supply a major parameter in cultivations requiring special process design strategies. In standard fed-batch cultivation, dissolved oxygen concentration inside a bioreactor is kept at certain level by pumping air and pure oxygen to the reactor. There are safety concerns over handling of oxygen especially in large scale. Therefore, there is a need to develop a production process under oxygen limited conditions.

*A P. pastoris* GS115 his4 strain under AOX1 promoter system expressing single-chain antibody fragment (scFv) was used in this study. Both methanol and oxygen parameters influence the production process and the objective was to find a robust production process. Fed-batch fermentations were performed in a 10 L scale bioreactor. The effects of lower oxygen level, glycerol feeding duration and specific substrate uptake rate on product formation were studied. The fed-batch fermentations were carried out in a bioreactor with basal salt media.

A process was developed for a scFv production in *P. pastoris*. The product levels of 3.5 g L\(^{-1}\) scFv in culture supernatant were achieved and production process was designed without additional need of pure oxygen thus relieving safety requirements and lowering the amount of methanol. The methanol uptake control strategy is beneficial for those products that suffer from degradation or modification during limiting feeding of methanol.
Enzyme-based glucose delivery system (EnBase) is a fed-batch-like method applicable to simple shaken cultivations. Since glucose-feeding rate can be adjusted by enzyme dosing to match the cultivation conditions (especially oxygen transfer rate and cell density), EnBase technology can provide a level of growth control, which is normally applicable only in bioreactor cultivations [1]. Additionally, glucose-limiting conditions create the possibility to maintain a favourable pH level by applying a balanced mixture of organic and inorganic nitrogen sources [2].

An efficient cultivation protocol was developed for *Pichia* and the new EnBase yeast medium composition, EnPresso Y Defined tablets. Since slow glucose-delivery does not prevent the induction of AOX1 promoter, cultivations were performed to assess the cell densities and product yields obtained with EnPresso Y Defined compared to mineral salt medium or the rich medium, BMMY. The developed cultivation protocol has shown an increase in cell mass and improved product yields while maintaining a favourable pH range. In these experiments several recombinant proteins cloned into methanol-inducible expression system showed significantly improved production with EnPresso Y Defined medium. Proteins, such as a plant-lectin binding protein, Mpv17 (human integral membrane protein), Erp18 (human endoplasmic reticulum protein), and fungal lipase showed 3- to 10-fold improved volumetric protein production. Optimisation of the cultivation protocol was performed using a GFP-tagged construct expressing human adenosine receptor A3 and this procedure revealed that the EnBase technology can also reduce the metabolic stress caused by methanol or by frequently changing conditions observed in shaken cultures. Interestingly, EnBase technology can improve the protein quality as observed when protein quantification determined by fluorescence signal is performed on the neat sample compared to the purified protein sample.

EnPresso Y Defined medium, which feeds low amounts of glucose throughout the cultivation, can provide stable conditions, which are ideal for protein production in *Pichia pastoris*. The major benefits include that the amount of the toxic inducer methanol can be minimized, there is no requirement for the change of cultivation medium and the medium can be easily prepared from pre-sterilized tablets. This cultivation protocol, which includes occasional addition of the glucose-releasing enzyme together with methanol addition, can be easily adapted to any vessel or shaking condition or to specialised processes, e.g., the stability of the (secreted) recombinant protein is pH-dependent.

References
Alternative secretion signals for *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* is becoming one of the most widespread hosts for recombinant protein expression [1]. Several properties of its phenotype are advantageous for protein production; in particular, the scarcity of endogenous secreted proteins allows secreting a product of interest to quasi-homogeneity in culture supernatant. In order to secrete a gene product, only recently alternative sequences to the widely used α mating factor (α-MF) from *Saccharomyces cerevisiae* became available [2]. A requirement for such a “second generation” of secretion signal is represented by being encoded by a short sequence, to facilitate the cloning process, and by a smoother expression and processing by *P. pastoris* secretion apparatus.

To identify novel – endogenous – secretion signals in *Pichia pastoris*, instead of starting from bioinformatic information, an empirical approach have been followed; previous studies in our lab, analysing *P. pastoris* cultures treated with the N-glycosylation inhibitor tunicamycin, indicated the presence of two distinct proteins released in the media on a pathway of active secretion, thus highlighting the possibility to identify two novel secretion signals. Upon MS-sequencing of these proteins and *in silico* characterization of their sequence, an N-terminal fragment, hypothesized to contain the secretion signal, was cloned upstream of the reporter gene for *C. antarctica* lipase B. Screening for high level of secretion in *P. pastoris*, both in deep well plate or shake flask cultivation, showed a high performance in secretion for both novel secretion signals when compared to α-MF-mediated secretion. The product characterization, moreover, showed a high level of consistency and homogeneity for the secreted product, as expected from endogenous secretion sequences. Further work involving the new secretion signals will be focus on the improvement of the knowledge on the proteins of origin, never characterized before, together with a deeper characterization of the processing steps in *P. pastoris*.

References
Optimization of methanol-free fermentation with 2nd generation AOX1 promoter variants

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As compared to the AOX1 wild type promoter or 1st generation AOX1 promoter variants, VTU Technology’s 2nd generation AOX1 promoter variants are derepressed earlier on common carbon sources like glucose and glycerol, i.e. at a higher concentration of these nutrients. Fermentation and feeding strategies were developed to benefit from this feature in methanol-free, glycerol-driven fermentations. The omission of methanol, despite most often reducing production levels, brings several advantages, e.g. avoidance of a toxic and explosive compound as well as lower oxygen consumption and heat production during fermentation. Multiple *Pichia pastoris* expression strains were cultivated in parallel following a standard as well as an alternative feeding strategy. The total fermentation time was reduced by applying an exponential feed rate without sacrificing productivity, thus improving the space time yield. Future optimizations are aimed towards further reduction of the overall process time and increased productivity.
Clonal Variation in recombinant *Pichia pastoris*

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The high volumetric productivities and purity of some secreted heterologous proteins has made the methylotrophic yeast *Pichia pastoris* a popular recombinant protein expression platform. However, these features appear to be protein specific and, even when specific productivity is relatively high, there appears to be a large amount of clonal variation, wherein clones from the same transformation can give significantly different titres. Companies often screen up to 1000 colonies to find the best secretor to overcome clonal variation, which is costly and time consuming. Identifying the underlying causes behind clonal variation might help to minimise the problem and reduce the screening effort.

Clonal variation is partly due to copy-number variation as, depending on the transformation method, this is known to vary over a wide range, giving occasional “jackpot” clones with >10 copies of the heterologous gene. However, in this study we have demonstrated that even in clones with single gene copies there can be a wide range of productivities. Twenty-three mut+ clones from a single transformation of GS115, using a vector containing the human serum albumin gene (HSA), were selected and confirmed as single copy by qPCR using the ACT gene as the housekeeping gene. HSA was chosen as a model protein due to its ease of expression in this host, with reported titres of greater than 10 g L$^{-1}$ [1]. Titre levels, measured using the Albumin Blue Fluorescence Assay (Active Motif, Belgium), varied from 2.5 mg L$^{-1}$ to 22.5 mg L$^{-1}$ HSA after 24 hour expression in microtitre plates. Once clear variation had been established nine strains were selected based on titre levels alone and split into three statistically distinct secretion categories; high, mid and low. Expression was carried out in baffled flasks for 24 hours after induction with methanol and RNA samples taken for microarray analysis and specific qRT-PCR of HSA transcripts and transcripts associated with the unfolded protein response. HSA titre levels were also determined with the fluorescence assay. Microarray analysis was done using an optimised, second generation, *P. pastoris* specific probe set. In this poster, the preliminary findings of this analysis will be presented and discussed in the context of their effect on protein secretion and the possibility for optimizing screening regimes.

Reference

Trick or treat – optimizing adaptation of *Pichia pastoris* to methanol in fermentation


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*Pichia pastoris* is increasingly recognized as an attractive host for the production of biopharmaceuticals, (diagnostic) enzymes and other proteins. Highest protein titers are usually obtained by expressing the target gene under the control of the inducible strong AOX1 promoter. Using a library of variants of this promoter, VTU Technology has successfully produced a broad range of target proteins at previously unseen levels.

In a typical fermentation for expression of a gene under the control of an AOX1 promoter variant, cells will first be grown on glycerol or glucose to generate sufficient amounts of biomass prior to induction using methanol. The initial hours of the production phase however represent a challenge to the cells as they have to adapt to the new substrate methanol. On a cellular basis this requires an entire re-arrangement of metabolic pathways, up-regulation of a completely different set of genes and massive proliferation of peroxisomes. Obviously, rapid adaption of the cells to the changing conditions has to be crucial for the final outcome of the entire fermentation process.

We have investigated different strategies to accelerate adaptation of *Pichia pastoris* to methanol as a substrate in fermentation using on-line analysis of the off-gas composition and off-line HPLC measurements. We show that a cut-off has to be found between challenging the cells to trigger rapid adaptation of the metabolism while still ensuring maximum cell viability by providing sufficient amounts of an easily metabolized carbon source like glycerol.
Whole-cell screening of functional CYP52s and reductases for heterologous expression in *Pichia pastoris*

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Cytochrome P450s (CYP450s) are heme-containing proteins, participating in stereo- and region-specific monooxygenation reactions, and capable of activating substrate’s inactive C-H bonds resulting into a hydroxylated product. CYP450s depend on NAD(P)H as a source of electrons, which are delivered to CYP450s by reductases, NAD(P)H-dependent redox partners; these two enzymes work in a synergic manner in crucial pathways, from detoxification of exogenous compounds to modifications of antibiotic structures. Such a unique enzyme system has raised much interest for its scientific value and industrial potential.

*Pichia pastoris* has become one of the most popular hosts for the expression of heterologous proteins, due to the ability to express the high levels of functional proteins. It has been proven as a successful host for expression of detectable amounts of active CYP450s from plant, animal, human and fungus.

To expand the screening potential for eukaryotic P450 determination (we focused our attention in particular to P450s from *Candida maltosa* and *Candida tropicalis*, belonging to the CYP52 family), simpler screening methods must be applied to detect activity in yeast cultures; using whole bacterial cells, total P450 levels have been detected in high throughput or medium throughput mode, using a straightforward dithionite/CO treatment of bacteria in microtitre plates. The same method could also be adapted to the yeast cells, if high levels of functional CYP450 expression are achieved. To the best of our knowledge, direct measurement of P450 levels in whole yeast cells in microplate format has not been reported to date. In the present study we have established *Pichia pastoris* whole-cell CO spectral determination in 96 well format, to screen for clones expressing functional heterologous CYP450s. Attempts were also made to use cytochrome C reductase assay with *Pichia pastoris* whole cells in 96 well format, to check the active expression of heterologous cytochrome reductases. Different CYP52s and their reductases have been chosen in these studies; a fast and reliable functional assay allowed us to define the best expression ratio for the two redox partners involved, providing quick results to develop an optimal *P. pastoris* strain for P450-based biotransformation.
A multistep strategy for selection of high diabody producers in *P. pastoris*.


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With the purpose of developing a proprietary *Pichia pastoris* expression system, Eurogentec has developed technological tools going from the construction of the expression system to the recovery of the protein of interest. These optimizations include the design of the cloning strategy, the high-throughput screening for selection of high productivity clones, the validation and culture optimization of selected clones in small-scale bioreactors, the scaling-up to a production volume and the recovery of the protein of interest from the supernatant using microfiltration.

The case study presented here are results obtained for the production of an antibody fragment (diabody) optimized for imaging purposes. Using Eurogentec’s *P. pastoris* expression platform, 10 different expression strain possibilities based on the combination of 3 major parameters (signal peptide, gene copy number and methanol utilization phenotype) are available. An initial single gene copy construct featuring his4 selection, methanol induction and using the α-factor signal peptide for protein secretion has been used for the transformation of *P. pastoris* NRRL Y-15581. The transformation strategy targets the production of hundreds of Mut+ clones.

Between 48 and 192 clones were screened for protein production using a high-throughput (HT) micro-fermentation system allowing on-line monitoring of the biomass and O2 consumption. The production yields were evaluated by HT SDS-PAGE analysis. Further selection of the best candidates was performed using a fed-batch fermentation process in down scale fermentors.

An experimental plan based on 4 critical induction parameters (temperature, biomass level, pulsed vs continuous methanol feeding and methanol concentration) was used to optimize the protein productivity in 5l bioreactors. This strategy generally increases the production yield by a factor 2 to 10. In this case study, yields of 1.7 g/l of diabodies were reached.

Due to the control of the different physical and chemical parameters (vessel geometry, Kla, agitator tip speed...) known to be critical in fermentation processes, a direct 10-fold scale-up was successfully performed (comparable fermentation profiles and protein production yields).

The protein of interest was concentrated by microfiltration with a yield higher than 90% and directly conditioned by diafiltration in a buffer compatible with the first step of the downstream processing.
Expression of a *Trichoderma reesei* endo-N-acetyl-β-D-glucosaminidase (EndoT) in *Pichia pastoris*

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Saprophytic filamentous fungi produce and secrete a variety of hydrolases, needed for the degradation of organic material. One of the best producers of such enzymes is *Trichoderma reesei*. Amongst the enzymes produced by *T. reesei*, an endo-N-acetylglucosaminidase-like activity, here called EndoT, was identified and purified [1]. This enzyme was found to responsible for a specific glycosylation pattern determined by the presence of only a single GlcNAc-residue onto the Asn of known N-glycosylation sites.

Enzymes acting on the chitobiose part of N-linked glycans, like endo-N-acetyl-β-D-glucosaminidases (e.g. Endo H) and N-linked-glycopeptide-(N-acetyl-beta-D-glucosaminy)-L-asparagine amidohydrolases (e.g. PNGase F) are important tools in the isolation and analysis of oligosaccharides from glycoproteins. Moreover, glycosidases that are able to deploy deglycosylation activities on a native protein (such as Endo H) have proven to be invaluable for the elucidation of the crystal structure from several glycoproteins. Purified *T. reesei* Endo T was proven to be able to act upon high-mannose and hybrid, but not on complex N-glycans from native proteins.

These characteristics made *T. reesei* Endo T into a useful tool to increase N-glycan homogeneity in glyco-engineered *Pichia pastoris* or for the production of single-GlcNAc modified glycoproteins, for e.g. crystallisation [2].

References
Expression and glyco-engineering of Alpha-galactosidaseA in *Pichia pastoris* for the treatment of Fabry disease

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Fabry disease is an X-linked recessive lysosomal storage disease arising from deficiency of the lysosomal enzyme Alpha-galactosidaseA (GLA). Since this enzyme catalyzes the removal of galactose on glycolipids in the lysosomes, globotriaosylceramide accumulates and causes chronic pain, cardiac diseases, liver and kidney failing. Currently, recombinant enzymes used for enzyme replacement therapy, are mostly produced in mammalian cell-lines. Since this method has some serious disadvantages such as high production costs, low effectiveness and real chance of viral infections, a different protein production system is preferable. Besides *E. coli* [1] and baculovirus-infected insect-cells [2,3], previous attempts have used *Pichia pastoris* [4] and *Saccharomyces cerevisiae* [5]. But for these latter organisms neither the yield nor the N-glycosylation profile was satisfactory.

Based on an och1 mutant *Pichia* strain with relatively homogenous N-glycosylation, we engineered a production process to obtain a high yield of GLA. First, we evaluated different cultivation temperatures and pH's and found that the production yield in shake flask culture doubled at 25°C vs. 28°C. Second, we evaluated the effect of the chemical chaperone deoxygalactonojirimycin (DGNJ) to facilitate/stabilize the correct folding of GLA. Whereas the addition of DGNJ to the culture medium greatly enhanced the production level of GLA shown in a *Yarrowia lipolytica* expression strain, we observed no such enhancement for *Pichia pastoris*. Finally, we induced the Unfolded Protein Response at the time of GLA induction through AOXI-driven expression of the spliced *Pichia pastoris* HAC1 [6], which resulted in a 10-fold increase of the secreted *Pichia pastoris* GLA activity. The final engineered strain produces GLA at a level comparable to mammalian cell production hosts. This work forms the basis for further work in high-cell density fermentation to further enhance the production yield.

To facilitate the targeting of lysosomal enzymes, N-glycans containing mannose-6-phosphate (M6P) groups are required. As a first step towards this goal, a *Pichia* strain able to produce highly phosphorylated N-glycans is made by overexpressing the endogenous *PNO1* gene. We introduced 2 extra copies of this gene, resulting in a strain in which the large majority of N-glycans are modified with either one or two Man-Pi-6-Man moieties.

References


VTU’s 2\textsuperscript{nd} generation AOX1 promoter variants for \textit{Pichia pastoris} bear a higher repression-threshold for concentration of glycerol (or glucose) thus enabling “methanol-free” protein production upon limited glycerol/glucose feed. In this “derepression status” no negative regulation of the promoter is taking place, thus promoting efficient transcription of the target gene(s) without additional induction by methanol. Although in most cases protein production levels are below a methanol-driven approach, in general 30-70\% as compared to the best methanol-induced process with 1\textsuperscript{st} generation promoters are reached for many target proteins. Major advantages of this new technology – besides abolishing toxic and explosive methanol as a substrate – are reduced oxygen consumption in fermentation and therefore clearly reduced heat production as well as a significant potential to reduce process times. Maximizing space-time-yields of these fermentation processes is however challenging as the interplay between growth, biomass concentration and repression of the promoter – which all depend on the substrate feed and all influence protein production – is complex. We have analyzed fermentation processes in terms of specific rates of biomass growth and product formation and have conducted systematic experiments to understand and optimize fermentation processes using VTU’s 2\textsuperscript{nd} generation AOX1 promoter variants in \textit{Pichia pastoris}. 
From Strain to Product: A fast approach in bioprocess development for recombinant protein expression in *Pichia pastoris*

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**Motivation**

*Pichia pastoris* is one of the most important host organisms for the recombinant production of proteins in industrial biotechnology. Beside the genetic engineering of strains methods for a fast early bioprocess development in order to increase the process understanding of the used strain are needed. To date, strain specific parameters, which are needed to set up feeding profiles for fed batch cultivations, are determined by time-consuming continuous cultures or consecutive fed batch cultivations, operated at different parameter sets.

**Results**

Here, we developed a novel approach based on fast and easy to do batch cultivations with methanol pulses enabling a more rapid determination of strain specific parameters, such as specific substrate uptake rate $q_s$, specific productivity $q_p$ and the adaption time ($\Delta t_{adapt}$) of the culture to the inducer methanol [1]. The strategy was applied to several *Pichia pastoris* phenotypes (Mut$^-$ and Mut$^+$) expressing different recombinant products [2]. Based on $q_s$, an innovative feeding strategy to increase the productivity of the recombinant product HRP was developed. Higher specific substrate uptake rates resulted in increased specific productivity, which also showed a time dependent trajectory. However, a dynamic feeding strategy, where the setpoints for $q_s$ were increased stepwise until a $q_{s,max}$, resulted in the highest specific productivity (Fig. 1). Besides the application of this novel approach on a single substrate system, we additionally examined the impact of $q_s$ on controlled mixed feed fed batch systems, where cell metabolism and product formation showed different mechanistic behaviors.

**Conclusion**

Our strategy describes a novel and fast approach to determine strain specific parameters of recombinant *Pichia pastoris* strains for the set up of fed batch regimes based on $q_s$. We furthermore show the potential of a novel dynamic feeding profile based on $q_s$ to boost recombinant protein expression in comparison to conventional strategies. Upcoming studies focus on further optimization of the productivity by controlled $q_s$ ramp feeding profiles and the modeling of the promoter kinetics to further enhance bioprocess understanding for recombinant protein production in *Pichia pastoris*.
Figure 1: Fed batch cultivations of *Pichia pastoris* on the single substrate methanol: A, specific substrate uptake rate profiles; B, specific productivities.

References


Improved processing of secretory proteins in *Pichia angusta* by sequence variation of the alpha mating factor prepro sequence

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The references in literature as well as databases are ambiguous about the exact start of human interleukin-6 (IL-6) – three possibilities for the initiation of the mature protein are described there. These three different variants of IL-6 varying in the exact initiation of the mature protein (A28, P29, or V30) were expressed in *Pichia angusta* using the *Saccharomyces cerevisiae* MFα prepro sequence instead of the homologous presequence. All three IL-6 variants were secreted but the processing by the Kex2 protease showed significant differences. V30-IL-6 showed correctly processed material but also a molecule species of higher molecular weight indicating incomplete processing of the MFα pro peptide. With P29-IL6 no correctly processed IL-6 was achieved, instead only the unprocessed pro form was found in the culture supernatant. In the case of Ala28-IL-6 100% correctly processed material were observed. N-terminal sequencing of this material revealed a start at V30 – obviously the first two amino acids (A28-P29) have been removed by a so far unknown protease. Thus expression of both A28-IL-6 and V30-IL-6 as MFα prepro fusion proteins resulted in the very same mature V30-IL-6, however, the ratio of correctly processed molecules was significantly improved in the case of A28-IL-6.

The expression of an MFα prepro-interferon α-2a (IFNα-2a) fusion protein in *P. angusta* leads to about 50% correctly processed molecules and 50% misprocessed forms which contain a part of the propeptide at their N-termini. To investigate whether the transfer of A30 and P29 of IL-6 between the propeptide and the start of the mature IFNα-2a can improve the processing we expressed a MFα prepro-AP-IFNα-2a in *P. angusta*. In analogy to IL-6 the processing of the new MFα prepro sequence variant during expression of the IFNα-2a showed distinct improvement. The MFα prepro sequence was efficiently processed and all high molecular isoforms observed in earlier experiments were eliminated.
*Pichia pastoris* as cell factory – Examining the secretory capabilities

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The unicellular yeast *Pichia pastoris* is of major industrial interest as a host for heterologous protein production. It can be cultivated to high cell densities in bioreactors reaching high titers of secreted protein and due to its eukaryotic nature, it is capable of performing complex posttranslational modifications. The list of proteins expressed and secreted successfully in *P. pastoris* is quite comprehensive, but particularly secretion of multimeric proteins sometimes poses challenges. Despite the high relevance of this microorganism as a host, only little is known about the secretory machinery and possible bottlenecks thereof.

This work focuses on the intracellular fate of a host of model proteins, some of which can be detected in the culture supernatant in very small quantities only. The model proteins include monomeric proteins that rely on correct disulphide bond formation as well as complex heterodimeric proteins of different size. Protein expression driven by the strong inducible *AOX1* promoter and the constitutively active *GAP* promoter is being evaluated. Elucidating the subcellular localizations of the model proteins is the aim of the first phase of this study. This will enhance the knowledge about possible bottlenecks and sinks along the secretory pathway of *P. pastoris*.
100% double site saturation by individual expression of 400 muteins in *P. pastoris*

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Cytochromes P450 constitute a source of interesting proteins. On the one hand, they play a pivotal role in all kingdoms of life, being involved in many biosynthetic, catabolic, and signaling pathways [1]. On the other hand, they could also serve as valuable and versatile biocatalysts because of their ability to hydroxylate non-activated carbons in a regio- and enantioselective way [2]. However, 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 *P. pastoris* for the efficient expression, laboratory evolution and rational engineering of P450s.

The current work is focused on investigating the catalytic properties of the cytochrome P450 2D6 (CYP2D6) towards testosterone, an atypical CYP2D6 substrate. An initial multi-site saturation mutagenesis library on the positions E216 and F483 was generated, since it was recently shown that these positions play a role in substrate specificity of CYP2D6 [3, 4]. Screening of this library revealed muteins with improved and new testosterone hydroxylase activities. Encouraged by these results, we decided to investigate in more detail, which amino acid residues on position E216 or F483 are responsible for higher testosterone conversion and which ones have an impact on the regioselectivity. Therefore, all possible 400 CYP2D6 variants have been generated, expressed in *Pichia pastoris* and tested for their testosterone hydroxylase activity. To achieve this in a minimal amount of time, tools for high-throughput such as a protocol for a *Pichia* transformation in the 96 well-format have been developed.

References

Characterization of *Pichia pastoris* Golgi and plasma membrane

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*Pichia pastoris* has reached a prominent status regarding expression of recombinant proteins and is worldwide used as a highly efficient cell system for the production of heterologous proteins. The ease of employing molecular biological tools comparable to *Escherichia coli* or the baker’s yeast *Saccharomyces cerevisiae* as well as high yield of cell biomass made *Pichia pastoris* a most suitable system for host protein expression and secretion. Besides practical convenience *Pichia pastoris* brings about major advantages such as posttranslational modifications which are similar to mammalian cells. For these purposes *Pichia pastoris* can be used in a broad variety of biotechnological and pharmaceutical applications. Despite the importance of *Pichia pastoris* in biotechnology, little information is available so far about the cell biology of this microorganism. This fact tempted us to intensify our studies on *Pichia pastoris* organelles with special emphasis on subcellular fractions involved in the classical secretory pathway. The Golgi accommodates many processes of post-translational protein modifications and can be seen as a major branching point within the secretory pathway. A detailed description of its structural composition will be of high value for fundamental understanding of this organelle. The plasma membrane is important for the protein secretory pathway because it is the last barrier on the way of polypeptides to be externalized from the cell. Here we present protocols to isolate *Pichia pastoris* Golgi and plasma membrane at sufficient yield and purity. GFP-hybrids of cis- and trans-Golgi marker proteins allowed intracellular tracking and provided an essential tool for achieving isolation and separation of cis and trans-Golgi compartments which then were analyzed in detail. Isolated plasma membrane fractions from cells grown on different carbon sources were biochemically investigated with emphasis on the composition of proteins and lipids (sterols, fatty acids and phospholipids). Electron microscopy supplemented these studies and provided information about the internal cell structure under different growth conditions. Similarities of *Pichia pastoris* and *Saccharomyces cerevisiae* became evident, although certain differences have to be kept in mind. Comparison of the two yeasts is important for understanding different properties and features and designing new strategies to improve *Pichia pastoris* strains for industrial applications.
Advanced bioprocess monitoring by implementation of Proton Transfer Reaction - Mass Spectrometry (PTR-MS) for measurement of volatile components in the bioreactor

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Common bioprocess conditions imply a gas-liquid-mixture with living cells as solid phase in a sterile environment which demands a great deal on sensor/analyser technology and design. On-line access to physiology relevant process variables, the ultimate request of process engineers, is still very limited as complexity of biological systems additionally constrains direct measurements. Volatile organic compounds in the gas/liquid phase either provided by medium and gas input or emitted by cells (exa-metabolome) represent a class of analytes accessible via analysis of the fermenter exhaust gas. The required high sensitivity for VOC measurements and a linearity range of multiple orders of magnitude are perfectly matched by the PTR-MS technology. The potential of PTR-MS based VOC measurements in bioprocess monitoring was assessed by using recombinant protein production processes with E. coli as model system.

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www.pichia2012.com
Pichia pastoris lipid droplet lipidome

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The industrial yeast *Pichia pastoris* is widely used as an experimental system for the heterologous expression of proteins. Despite the extensive commercial use, fundamental biochemical, cell biological and molecular biological knowledge about this microorganism is rare. As a striking example, *Pichia pastoris* lipids have not yet been studied in much detail although it is obvious that knowledge about this class of biomolecules is highly important to understand the impact of biological (organelle) membranes on protein targeting and intracellular translocation of heterologously expressed proteins. Another important compartment with respect to lipid metabolism are lipid particles/droplets. In lipid droplets, non-polar lipids (triacylglycerol, steryl esters) are stored, but in addition a number of enzymes mainly involved in lipid metabolism are located to this compartment. To investigate the molecular components of *Pichia pastoris* lipid droplets in some detail we started to analyze its lipidome. For this purpose, *Pichia pastoris* lipid droplets were isolated at high purity and analyzed for non-polar lipids, phospholipids, fatty acids and sterols. The data obtained are consistent with the current general knowledge about lipid droplets from other yeasts, especially *Saccharomyces cerevisiae*. However, some distinct features of lipids from *Pichia pastoris* lipid droplets are the high amount of unsaturated fatty acids and the prevalence of triacylglycerols over steryl esters. To shed more light on the biogenesis of *Pichia pastoris* lipid droplets we generated and characterized mutant strains lacking triacylglycerols and/or steryl esters synthesizing enzymes and described the contribution of these enzymes to neutral lipid formation. These results are the basis for further molecular biological investigations of non-polar lipid metabolism and for a broader view of organelles from *Pichia pastoris*.

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Engineering *Pichia stipitis* for xylitol production from the diluted acid extract of empty palm fruit bunch fiber

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Xylose is a major constituent of lignocellulosic biomass, and the efficiently fermenting xylose is important for production of biofuels and chemicals in biorefinery [1]. *Pichia stipitis* is a native xylose fermentable yeast and presents several interesting feature as a promising industrial hosts [2,3]. In this study, to produce xylitol from xylose derived from the diluted acid extract of empty palm fruit bunch fiber (EPFBF), a wide type of *P. stipitis* CBS5773 was engineered by the disruptions of a gene coding xylitol dehydrogeanse (xyl2) or xylulokinase (xyl3) involved in xylose metabolism using the *kanMX-loxP* cassette. The mutants were selected by the responsibility for resistance to G418, and the correct integrations of the gene disruption cassette were confirmed by PCR. The xylitol productions of the gene deletion strains were tested in the acid extract of EPFBF supplemented with yeast extract/peptone as a nitrogen source. The productivity of xylitol converted by NADPH-dependent xylose reductase (XYL1) was higher in the mutated strains than in the wild-type. In fermentation experiments using glycerol as a co-substrate, the mutant strains produced 13 g/L of xylitol, 10 times higher than that in the wild-type strain, and showed a higher volumetric productivity 0.1 g xylitol l\(^{-1}\) h\(^{-1}\) under aerobic culture condition.

References
Lipidome of subcellular fractions from the yeast *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* is the most popular expression system for the production of heterologous proteins in biotechnology. Although cell organelles play obviously an important role in this process, little is known about cell biological and biochemical properties of subcellular fractions from *Pichia pastoris*. For this reason, we started a systematic approach to isolate and characterize organelles from this yeast. Cell fractionation procedures were adopted from protocols originally designed for *Saccharomyces cerevisiae* and optimized to achieve highly purified organelles. Due to the lack of appropriate antibodies GFP-tagged marker proteins were constructed and used for organelle quality control. Analysis of organelles is focused on membrane components with emphasis on protein and lipid patterns. Organelle lipidomics include analysis of phospholipids, fatty acids, sterols and non-polar lipids. Current investigations are focused on the characterization of mitochondrial and microsomal membranes. Work is in progress to construct mutant strains compromised in key steps of lipid biosynthesis. These strains will be used to manipulate the composition of subcellular membranes and study possible effects on protein secretion.

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Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway


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The biotechnologically important yeast *P. pastoris* can live on methanol as the sole carbon source. The first step of the methanol utilization pathway is catalyzed by alcohol oxidases, AOX1 and AOX2. The strongly inducible promotor of *AOX1* is often used for the expression of recombinant genes. Knockout of the natural *AOX1* yields strains with Mut* (methanol utilization slow) phenotype which have to rely on the weaker AOX2 activity for methanol utilization [1] but which sometimes are better producers of recombinant proteins than Mut+ wildtype strains, *e.g.* [2]. The methanol utilization pathway is directed towards dissimilation by the activity of a formaldehyde dehydrogenase FLD1. Assimilative methanol utilization is initialized by the activity of a dihydroxyacetone synthase DAS1 or DAS2, or putatively also by a transketolase TKL1 [3]. We show the favorable methanol utilization phenotype for the production of horseradish peroxidase and the influence of co-overexpressed DAS1, FLD1 or TKL1 on the production of horseradish peroxidase and *Candida antarctica* lipase B by these engineered strains [4].

References

New expression systems for heterologous protein production in 
*Pichia jadinii* and *Candida utilis*

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*Pichia jadinii* is known as the teleomorphic form of the polyploid yeast *Candida utilis*, which is classified as GRAS (*generally recognized as safe*). *C. utilis* already represents an industrially important yeast because of its ability to assimilate a number of different carbon and nitrogen sources [1]. Furthermore, growth of *C. utilis* is hardly affected by extremes in pH, and being Crabtree-negative it does not produce ethanol in aerated cultures, which limits growth of other yeast species such as *Saccharomyces cerevisiae*. Initially, *C. utilis* was used as food yeast to produce single cell protein in high quality from cheap, biomass-derived waste substrates [2].

We developed a set of novel expression vectors for heterologous gene expression in *C. utilis*. These vectors contain the *sat1* gene under the control of the *Candida albicans ACT1* promoter for selection of transformants on nourseothricin-containing media. Furthermore, we have established the *C. utilis* genome sequence, which allowed us to identify a number of strong promoters of *C. utilis* genes including *TDH3*, *PGK1* and *PMA1*, which were used to drive heterologous gene expression. Promoter efficiencies were evaluated using genes encoding green fluorescent protein and *Candida antarctica* lipase B as reporters. We report that the nourseothricin-based expression plasmids can be used in the four yeast species *C. utilis*, *P. jadinii*, *S. cerevisiae* and *C. albicans*.

References


Bulk expression of a potential anti-HIV1 microbicide? *Pichia pastoris*, the right tool for the job


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Yearly the HIV/AIDS epidemic affects ~2.5 million people with a global estimate of more than 33 million HIV-positive people worldwide. With few promising results in clinical vaccination trials, the focus has shifted towards the development of highly specific, potent and cost-effective microbicides to prevent HIV-1 transmission, for example by blocking CCR5-mediated HIV entry[1]. The protein 5p12RANTES, an analogue the native CCR5-ligand CCL5 was identified by phage display as a potent inhibitor of HIV-1 entry both *in vitro* and *in vivo* [2]. Unlike other CCL5 analogs that have been developed, 5p12RANTES only contains natural amino acids making it amenable for recombinant production which can contribute to the development of 5p12RANTES as an affordable microbicide. We report on the engineering of a *Pichia pastoris* strain expressing high amounts of intact 5p12RANTES under shake-flask conditions. We evaluate the effect on protein expression of copy number amplification and inducible overexpression of *P. pastoris* HAC1 gene [3] and touch on their relevance under fermentation conditions.

References
Combined use of flow cytometry to quantify the physiological state of *P. pastoris* in high-cell-density fedbatch cultures

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Matching both the construction of a recombinant strain and the process design with the characteristics of the target protein has the potential to significantly enhance bioprocess performance, robustness, and reproducibility. The factors affecting the physiological state of recombinant *Pichia pastoris* Mut+ (methanol utilization-positive) strains and their cell membranes were quantified at the individual cell level using a combination of staining with fluorescent dyes and counting by flow cytometry.

Cell vitalities were found to range from 5 to 95% under various process conditions in high-cell-density fedbatch cultures, with strains extracellularly producing either porcine trypsinogen or horseradish peroxidase. Impaired cell vitality was observed to be the combined effect of production of recombinant protein, low pH, and high cell density. Vitality improved when any one of these stress factors was excluded. At a pH value of 4, which is commonly applied to counter proteolysis, recombinant strains exhibited severe physiological stress, whereas strains without heterologous genes were not affected. Physiologically compromised cells were also found to be increasingly sensitive to methanol when it accumulated in the culture broth. The magnitude of the response varied when different reporters were combined with either the native AOX1 promoter or its synthetic variant, which differ in both strength and regulation.

Finally, the quantitative assessment of the physiology of individual cells enables the implementation of innovative concepts in bioprocess development. Such concepts are in contrast to the frequently used paradigm, which always assumes a uniform cell population, since differentiation between the individual cells is not possible with conventional methods for process monitoring.
PEGylate the smarter way: site-specific PEGylation of a single-domain antibody (sdAb) via chemical cleavage of intein-fusion proteins secreted from *Pichia pastoris*

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PEGylation still represents the method of choice for the *in vivo* half-life extension of therapeutic proteins. Performing this chemical modification at one specified site of the target protein is highly desirable to minimize the significant losses in biological activity often associated with non-selective PEGylation techniques. In addition, the production of a single homogeneous PEGylated species greatly simplifies analytical effort and regulatory documentation.

A technology that is proving particularly attractive for site-specific protein PEGylation, is the C-terminal PEGylation approach developed by Almac. In this procedure, C-terminal hydrazide or aminoxy derivatives of the target protein are generated through chemical cleavage of the corresponding intein fusion protein. The C-terminal hydrazide (or aminoxy) protein is then site-specifically PEGylated in high yield, under aqueous conditions, through chemoselective reaction with benzaldehyde-PEG derivatives. Using this robust approach, highly active homogeneous C-terminal PEGylated derivatives of a variety of different proteins have been produced.

Here we present the successful application of this site-specific PEGylation technology to proteins (single domain antibodies) produced through secreted expression from *Pichia pastoris*.

After screening and fermentation, an sdAb-Intein-6xHIStag fusion was produced at high titer as secreted protein. Direct IMAC purification of the culture supernatant allowed for quick and reliable supply of purified product. The IMAC-purified intein-fusion protein was then chemically cleaved, at the sdAb-intein juncture, to generate the corresponding C-terminal hydrazide or aminoxy derivative of the protein. After buffer exchange, the derivatised-protein was simply reacted with a benzaldehyde derivative of PEG(20K) to afford site-specific C-terminal PEGylation in high yield.

The secreted expression of intein-fusion proteins from yeast, in combination with C-terminal PEGylation methodology, provides a facile approach to the production of homogenous site-specifically PEGylated proteins, and consequently the development of protein therapeutics.
An efficient approach to the design of processes for enzyme production with recombinant *Pichia pastoris*

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Optimum conditions for the production of a recombinant protein differ according to the target product and promoter used. As a rational basis for the design of high productivity processes, the relationships between product formation and specific growth rate ($\mu$) need to be established empirically for every new strain construct at a range of conditions including $T$, $pH$ and medium composition. To speed up and rationalise this highly demanding first phase of all biotechnological process development, an approach for generic process design has been developed.

By systematically varying the specific growth rate, the entire production range was covered in a single experiment. In theory, linearly increasing and decreasing the rate of substrate addition in fedbatch cultivation enables a range of $\mu$-values to be analysed repeatedly while the age of the cells and the biomass concentrations increase continuously. This method was applied to a *Pichia pastoris* strain overproducing lipase B from *Candida antarctica* under the control of a synthetic AOX1 promoter variant, which is inducible in the presence of glycerol alone.

Finally, a process resulting in more than 6 g/l of enzyme was established with glycerol as the sole carbon source, and knowledge for optimisation of the strain design was efficiently generated.
High level protein expression in *Pichia pastoris* combining synthetic promoters and synthetic genes

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Within the last years we developed a new toolbox to further enhance and facilitate protein expression by *Pichia pastoris*. Herein we demonstrate the beneficial combination of several of these tools for protein expression. A set of 48 *TrCBH2* genes was synthesized distinguished by synonymous codon substitution [1]. This set was expressed under the control of the wild type P(GAP) as well as newly generated synthetic promoters P(De2) and P(En) [2]. In micro-scale screening single copy activity levels were determined by released cellobiose concentration and varied from undetectable to ~500% of the native *TrCBH2* activity. Fed-batch cultivations were accomplished to compare the employed promoters and showed a superior performance of the synthetic P(De2) regarding the protein yield, enzyme activity and the transcript level. The strong P(De2) can be tightly regulated by varying glycerol concentrations and there is no need to use methanol in the fermentation process. Additional fed-batch cultivations were performed to confirm the micro-scale results. Therefore, strains with varying copy numbers [3] expressing an improved *TrCBH2* gene variant under the control of P(De) were employed. Although no methanol was used for induction we obtained more than 15 g/L of secreted *TrCBH2*. Concluding, we were able to improve extracellular cellulose production by *Pichia pastoris* with the help of synthetic promoters and synthetic codon optimized genes.

References
Optimization of diploid *Pichia pastoris* strains for the expression of full-length monoclonal antibodies


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Alder Biopharmaceuticals has developed a rapid, robust technology platform for the production of full-length humanized monoclonal antibodies in *Pichia pastoris*. This expression system captures all the attributes provided by this host (e.g., low cost, short cultivation times, strain stability) while eliminating product development risk associated with untested expression systems. Here, a novel approach for the generation of diploid *P. pastoris* strains expressing full-length antibodies with increased yield and quality is described. First, haploid strains containing multiple copies of either heavy or light chain genes are generated. Second, the transformed haploids are mated to produce a panel of diploid strains. These strains contain varying copy numbers of the heavy and light chain genes for the expression of the full-length antibody molecule. The impact of gene dosage of heavy and light chains on antibody expression and quality was then evaluated. Increased copy number led to two- to three-fold higher antibody yields by the strains. In addition, modulation of heavy to light chain ratios led to a reduction in the abundance of the main product-associated variant by more than 80%, resulting in secreted antibody of comparable quality seen by mammalian production platforms. Alder’s Mab Xpress platform has and continues to supply therapeutic antibodies for extensive clinical trial programs.
Dynamic metabolic modelling of *Pichia pastoris* cultures on glycerol based medium in bioreactor

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On the basis of metabolic structure and mass balances analysis, a macroscopic model was constructed for *Pichia pastoris* cultured on glycerol based medium to simulate and predict cell growth during both batch and fed-batch cultures with a proposed regulator for biomass activity. In addition to direct state variables (i.e. biomass, glycerol, and ammonia), CER and OUR calculated from exhaust gas analysis were also included in the model. The model was directly and crossly validated with 5 experimental cultures started in batch mode and followed in glycerol fed-batch mode. Model parameters were identified with confidence intervals and data consistency was checked between predicted and measured CERs. Parameter sensitivity analysis showed that the model outputs were mostly sensitive to parameters while glycerol concentration in bioreactor fell to the Monod saturation constant $K_{\text{gly}}$ (1.05±0.42 mmol/L in this work). In a word, the model presented here is valuable for biomass online estimation, regulating glycerol concentration (around 3~6 mmol/L) that would maximize biomass productivity and avoid catabolite repression of *AOX1* promoter during the transition from biomass growth to methanol induction.
Identification and characterization of novel promoters in *Pichia pastoris*

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*Pichia pastoris* is a well established protein production host; however the number of available strong promoters is limited. In addition, many promoters are dependent on methanol.

In order to identify new strong expressed genes and their respective promoters of *P. pastoris*, gene expression patterns were analysed with DNA microarrays. The potential promoter regions were cloned into a *P. pastoris* expression vector, which carries either an enhanced green fluorescent protein (eGFP) or human serum albumin (HSA) as a reporter gene and transformed in into a *P. pastoris* strain. Four out of six identified promoters showed expression in shake flask screenings with both reporter genes. One of these promoters even showed stronger eGFP expression and nearly the same HSA expression compared to clones with the GAP promoter under the same conditions. Fed batch fermentations could prove the superior properties of this promoter. Data of screenings and fermentations will be presented.
Pichia and HSA (-fusions) – a love story

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Only one organism secretes human serum albumin to higher concentrations than Pichia pastoris does: the native host. With 18 g/L of HSA in culture supernatant after methanol-driven bioreactor cultivation, this protein titer constitutes one of the highest results in the history of VTU Technology’s application of 1st generation AOX1 promoter variants. 2nd generation AOX1 promoter variants from VTU also delivered double-digit g/L levels of HSA in glycerol-driven fermentations.

As an obvious strategy, fusion of payloads to HSA might take advantage of the inherent high secretion rate of HSA. Cytokines or scaffolds fused to the N- and/or C-terminus of human serum albumin resulted in methanol-induced secretion levels of 7 – 20 g/L, proving the applicability of HSA as a highly efficient secretion carrier protein.
Fluorescence Microscopy of *Pichia pastoris*: Revealing Bottlenecks in Recombinant Protein Secretion

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Yeasts like *Pichia pastoris* have been proven to be valuable hosts for heterologous protein production. However, secretion of the target protein is very often the limiting step in protein production. To better understand these limitations we analyzed the secretion behavior of different model proteins: human serum albumin, which is readily secreted by *P. pastoris* in high quantities, in contrast to complex antibody fragments, which are more challenging for the host cell. The distribution of intracellularly retained recombinant protein was studied by confocal laser scanning microscopy. Immunofluorescent staining as well as fluorescent fusion proteins were used to identify bottlenecks in secretion. To follow the recombinant protein on the way through the secretory pathway, subcellular compartments involved in secretion were detected, such as endoplasmic reticulum, Golgi apparatus, COP-II vesicles and secretory vesicles. Thus, it was shown that cells secreting large amounts of recombinant protein exhibit a different intracellular pattern compared to low secreting cells. Furthermore, a comparative study with the well established mammalian CHO system confirmed the intracellular pattern of good secretors.

Taken together, this study leads to a better understanding of host cell physiology and helps to decipher additional physiological bottlenecks impeding heterologous protein production in *P. pastoris*.
A new latex agglutination test for surra based on recombinant *Trypanosoma evansi* RoTat 1.2 variant surface glycoprotein expressed in the *Pichia pastoris* GlycoSwitch™ M5 strain

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Surra is an infectious disease caused by the protozoan parasite *Trypanosoma (T.) evansi*. It affects a large spectrum of wild and domesticated animal species in Africa, the Middle East, Asia and Latin America. The principal host species varies geographically, but camels, horses, buffaloes and cattle are particularly affected, although other animals, including wildlife, are also susceptible. The parasite is transmitted mechanically by bloodsucking flies such as *Tabanidae* and *Stomoxys* species and occasionally by vampire bats in Latin America. It causes severe anemia, oedema, immunosuppression and various neurological disorders resulting eventually into the death of the affected animals. Hence, surra leads to serious economic losses to the farmers in terms of morbidity, mortality, abortion, infertility, reduced milk yield, costs for trypanocides and restriction of export of animals into non-endemic regions.

The serodiagnosis is based on an antibody detection assay using the predominantly expressed *T. evansi* variant surface glycoprotein (VSG) RoTat 1.2. Native antigens are therefore purified from bloodstream form *T. evansi* grown in rodents. Several antibody detection assays have been developed, including a direct and an indirect agglutination test (CATT/*T. evansi* and LATEX/*T. evansi* respectively). Testing for surra is compulsory for import/export of susceptible animal species and for this purpose the card agglutination test for trypanosomiasis, CATT/*T.evansi*, is the test recommended by the World Animal Health Organization.

To avoid the sacrifice of laboratory rodents for antigen preparation, an N-terminal fragment of RoTat 1.2 VSG was expressed recombinantly in the *Pichia pastoris* GlycoSwitch™ M5 strain which mimics the trypanosomal N-glycosylation pattern. The recombinant protein was His tag purified yielding 20 mg purified recombinant RoTat 1.2 VSG per liter cell culture. ELISA on sera of 25 experimentally infected goats and 185 camels (93 naturally infected and 92 uninfected camels) confirmed the diagnostic potential of the recombinant RoTat 1.2 compared with its native counterpart.

The ELISA test however, is not applicable in the field, where a simple, fast, accurate and individual test with a high sensitivity and specificity is required. For serodiagnosis of surra, a latex agglutination test has several advantages. It can be performed on any host species avoiding the need to use different conjugates for each susceptible host like horse, camel, dog etc. Furthermore, the same latex reagent can be incorporated either in a single or in a mass screening format test. Thus, the purified, recombinant RoTat 1.2 VSG was covalently coupled to 0.8 µm polystyrene LATEX particles and tested against the same goat and camel sera as above. The LATEX/recRoTat 1.2 showed a sensitivity of 98.28% and a specificity of 98.31% suggesting that the recombinant RoTat 1.2 VSG can be used as an alternative for native RoTat 1.2 for diagnosis of surra, thus eliminating the use of laboratory animals for antigen production.
Identification and isolation of GPI anchored proteins from *Pichia pastoris* and their potential for surface display of recombinant proteins

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Cell surface display of proteins is of big advantage for a variety of biotechnological applications, for instance directed evolution, screening of antibody libraries or use as a whole cell biocatalyst. A *Pichia pastoris* cell surface display system was developed using different anchors. BLAST searches of the *Pichia pastoris* CBS7435 genome (Küberl et al., 2011) based on *Pichia angusta* (*Hansenula polymorpha*) cell wall anchor gene sequences (Choi et al., 2002) resulted in 8 putative GPI anchored proteins, which were tested with *Bacillus subtilis* levanase (Schörgendorfer et al., 1988) as a model system. A FLAG-tag was placed between the levanase/anchor fusions. The resulting fusion proteins were surface displayed using *Saccharomyces cerevisiae* pre-pro alpha factor signal sequence under the control of the AOX1 promoter. All constructs were targeted to integrate at the aox1 locus and confirmed by MutS screening to have a consistent comparison between the potential of different anchors for surface display. Immunofluorescence microscopy showed uniform localization of levanase protein on the *P. pastoris* cell surface. Levanase activity was monitored by glucose UV hexokinase assay (Dipromed GmbH).

References


Improving the biophysical properties of full-length IgG – inspired by *Pichia pastoris*

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Antibodies and their derivatives have found a broad range of applications, from basic research to medical therapy. As part of their functions relies on binding to Fcγ receptors and therefore on the N-linked glycosylation in their CH2 domain, currently all approved monoclonal antibodies are produced in mammalian cell lines. Recently, however, *Pichia pastoris* has also been engineered to introduce complex, human-like glycosylation [1, 2] - thus expanding the range of possible production hosts to this yeast system. Here, we compare the influence of the choice of production system (mammalian HEK 293 cells vs. *P. pastoris*) on the biophysical properties of the expressed antibodies.

In our analysis of immunoglobulin G molecules (IgGs) of identical amino acid sequence but produced in either of these systems, dramatic differences in their aggregation susceptibilities were encountered. The antibodies produced in *P. pastoris* were much more resistant to aggregation under many conditions, a phenomenon found to be mainly caused by two factors. First, the mannose-rich glycan of the IgG from *P. pastoris*, while slightly thermally destabilizing the IgG, strongly inhibited its aggregation susceptibility, compared to the complex mammalian glycan. Second, on the *P. pastoris* produced IgGs, amino acids belonging to the α-factor pre-pro sequence were left at the N-termini of both chains due to imperfect processing by the enzyme dipeptidyl aminopeptidase A (encoded by STE 13). These additional residues proved to considerably increase the temperature of the onset of aggregation and reduced the aggregate formation after extended incubation at elevated temperatures. The attachment of these residues to mammalian IgGs confirmed their beneficial effect on the aggregation resistance – indicating the transferability of this finding. Secretion of correctly processed IgGs in the yeast system became also possible after systematic engineering of the precursor proteins and the processing site. This allowed the definition of optimal sequence composition for either aggregation-resistant or correctly processed IgGs, respectively.

Taken together, the presented results will be useful for the successful production of full-length IgGs in *Pichia pastoris*, give indications on how to engineer aggregation-resistant IgGs and shed new light on potential biophysical effects of tag sequences in general.

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Production of glucoamylase enzyme variants in *Pichia pastoris* expression systems

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Glucoamylases are glycoside hydrolases which catalyze the releasing of free glucose from the non-reducing ends of raw starch and soluble oligosaccharides. They are typically microbial enzymes classified in family 15 of glycoside hydrolases. The majority of glucoamylases are multidomain enzymes consisting of a catalytic domain (CD) often bounded to a non-catalytic starch-binding domain (SBD) by a serine- and threonine-rich highly O-glycosylated linker region (LR). Fungal glucoamylases are industrially very important enzymes widely applied in large amount in the manufacture of glucose and fructose syrups. For industrial use these enzymes are typically produced by filamentous fungi expression systems by several fermentation methods.

The goal of our studies was to produce different domain variants of *Aspergillus niger* glucoamylase in yeast expression system. *Pichia pastoris* strains were created for secreted production of glucoamylase enzymes fused to the α factor signal peptide of *Saccharomyces cerevisiae*. The constitutive GAP and the methanol inducible AOX1 promoter of the host was used to drive the expression of the different enzyme variants. Gene of the wild type *A. niger* glucoamylase was optimized for expression in *P. pastoris* and prepared by *in vitro* gene synthesis. DNA fragments encoding the CD, CD-LR and CD-LR-SBD enzyme variants was cloned in *P. pastoris* expression vectors (pGAPZA, pPIC9K). These expression constructs were integrated into the genome of the GS115 and KM71 host strains. Numerous transformant yeast cell lines were screened by growing on starch or high concentration geneticin containing plates for isolation high level constitutive or inducible protein producer *P. pastoris* strains. The enzyme producer properties of the selected strains grown in shake-flask cultures were tested and compared.
Translation of message RNA (mRNA) is a critical phase of protein expression and, therefore, key in enhancing the yield of commercial proteins. ProteoNic uses its leading expertise in optimizing mRNA usage to enhance protein production yield. Our experimental data indicate that a 50-fold higher ribosomal binding affinity is caused by the improved interaction between ribosomal and mRNA equipped with ProteoNic’s Translation Enhancing Elements (TEE-s). Proteonic has developed specific TEE-s which can be applied in different host systems, ranging from fungi and yeast to mammalian cells as well as plants. Here, we provide an example of the use of one ProteoNic’s TEE-s that positively increases the protein expression yields in the *Pichia pastor* platform.
Production and Processing of Interferon in *Pichia* - A Cut Above

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*Pichia pastoris* is being increasingly embraced by the biopharmaceutical industry as an expression system for therapeutic proteins. However, besides the goal of achieving high titers, also quality issues like correct N-terminal processing or protein homogeneity represent a challenge with certain proteins.

We report high level secreted expression of human Interferon alpha 2a (hIFNa2a) in *Pichia pastoris*. The sequence of this protein features a peculiarity, namely a disulfide-bridge involving the cystein at its very N-terminus which has been reported to cause incomplete cleavage of the signal leader peptide, presumably due to steric hindrance of the processing protease(s).

In initial experiments we have indeed observed a heterogeneous product in the supernatant of strains expressing hIFNa2a. Capillary electrophoresis revealed two major forms present in a 1:1 ratio and one less abundant variant. Through sequence analysis using mass spectrometry we have identified the expression products as native hIFNa2a and two defined variants bearing an 11 and 14 amino-acid extension at the N-terminus, respectively. Separation of these closely related forms in downstream processing would apparently be tedious and the loss of 50% of the expressed protein could clearly not be tolerated from an economic point of view.

The problem of N-terminal heterogeneity can partly be overcome by overexpressing KEX2 protease from *S. cerevisiae* and – surprisingly – also (synthetic) *P. pastoris* protein disulphide isomerase (PDI) in hIFNa2a secreting strains. Cleavage of the leader peptide is highly improved through co-expression of either of these helper proteins and correctly processed hIFNa2a is found in a 15-fold excess over the variants with non-native N-terminus.

In a second strategy we have expressed a chimeric protein of human serum albumin (HSA) fused to the N-terminus of hIFNa2a via a cleavable linker. As observed also for other examples, the HSA-fusion protein could be expressed in excellent yields. The HSA-hIFNa2a fusion was purified and treated *in vitro* with a protease giving a clean cut at the C-terminus of the linker peptide, i.e. at the N-terminus of hIFNa2a. The liberated protein was apparently homogeneous in capillary electrophoresis and mass spectrometry revealed one single form exhibiting the correctly processed N-terminus.

Furthermore, *in vivo* cleavage by co-expression of the protease also delivered a clean cut, releasing homogeneous hIFNa2a with native N-terminus, as proven by MS.
Making synthetic proteins in *Pichia pastoris*

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*Pichia pastoris* strains auxotrophic for methionine or the aromatic amino acids were supplemented with various methionine, tyrosine, phenylalanine, or tryptophan analogs during the expression of *Candida antarctica* lipase B. In this way, synthetic CalB variants were produced that contained the amino acid analogs at multiple positions specified by the codons for the related standard amino acid. The variant proteins were secreted into the growth medium, which facilitated their purification by simple dialysis and ultrafiltration. Analysis of the catalytic properties of the isolated enzymes revealed an improved lipase activity of the norleucine variant. The CalB variants containing fluorophenylalanine or fluorotyrosine showed prolonged shelf life. Our findings emphasize the suitability of *Pichia pastoris* as an expression host for synthetic proteins with non-standard amino acid substitutions at multiple sites.
Random mutagenesis in *Pichia pastoris* by resistance cassette transformation

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For many recombinant proteins produced in *Pichia pastoris*, functional secretion from the cell is one of the limiting factors in high-yield production and a challenging step to manipulate. In many cases, trial-and-error approaches are followed to obtain higher yields of a specific product, as not all the factors involved in secretion are known yet. Potential secretory bottlenecks could occur on the levels of transcription and translation, during folding, post-translational processing and sorting into vesicles taking place in the endoplasmic reticulum and Golgi compartments, or during the cargo's passage through the cell wall.

My work aims to unravel factors involved in the secretion mechanism by random insertion of a linear DNA cassette. The effect on the secretion of reporter enzymes is analyzed in subsequent screening experiments.

A linear DNA cassette was constructed that harbors the Zeocin resistance marker, as well as an *E. coli* origin of replication sequence that can be used to recover the sequence surrounding the integration site. Mutagenesis is done by transforming yeast with a high concentration of the amplified cassette, resulting in a satisfactory number of transformants.

The analysis of several cassette integration sites by genome-walking methods revealed that the integration occurs predominantly without degradation of the cassette. Furthermore, we analyzed the target site specificity of the integration event by sequencing and Southern blotting experiments. The integrated cassette did not show any insertion site bias underscoring the feasibility of the approach.

The colonies generated by integration of the cassette are currently screened for altered secretion level of two reporter enzymes, alternative pig liver esterase (APLE) and horseradish peroxidase (HRP).
(+)-Nootkatone production in yeasts

Wriessnegger T. (1), Augustin P. (1), Emmerstorfer A. (1), Müller M. (2), Kaluzna I. (2), Machereux P. (1,3), Schwab H. (1,4), Pichler H. (1,4)

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Membrane-attached cytochrome P450 monooxygenases (CYPs) are versatile and industrially important enzymes, playing major roles in drug metabolism as well as in the production of fine chemicals, pharmaceutical compounds or flavors and fragrances. However, a number of limitations have restricted their use in industrial processes including substrate specificity, the association of the enzymes with membranous structures, the co-expression of a P450-reductase, the need for a complex system of cofactor regeneration and low activity.

Upon expression in S. cerevisiae, the plant cytochrome P450 enzyme CYP71D55 from H. muticus (HPO) has been shown previously to hydroxylate the sesquiterpene (+)-valencene, a natural aroma compound of citrus fruits, yielding the intermediate product trans-nootkatol. Trans-nootkatol can be further converted to the highly sought-after grapefruit flavor (+)-nootkatone [1]. In this study, the yeasts P. pastoris and S. cerevisiae were used for comparative expression studies of HPO in combination with a cytochrome P450 reductase (CPR) from A. thaliana. Co-expression of HPO and CPR in both yeast systems was monitored by CO-difference spectra measurements, Western Blot analysis and activity assays, respectively. Intracellular production of (+)-valencene by co-expression of valencene synthase resolved the uptake-problem of the hydrophobic substrate (+)-valencene. P. pastoris whole-cell biotransformation of (+)-valencene resulted in the production of trans-nootkatol, which was subsequently oxidized to (+)-nootkatone by an intrinsic P. pastoris activity and additional over-expression of a P. pastoris alcohol dehydrogenase significantly enhanced the (+)-nootkatone yield. Thus, the yeast P. pastoris represents an advantageous and valuable whole-cell system for the stereo- and regioselective hydroxylation of (+)-valencene and, therefore, the production of (+)-nootkatone.

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