

Ex. 1029 Ahmad 2014

Applied and Microbiology Biotechnology

Volume 98 Number 12 June 2014

Mini-Reviews

Biosynthetic trends and future aspects of bimetallic nanoparticles and its medicinal applications
S.M. Roopan · T.V. Surendra · G. Elango · S.H.S. Kumar 5289

Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production
M. Ahmad · M. Hirz · H. Pichler · H. Schwab 5301

Role of autophagy and its significance in cellular homeostasis
M.U.F. Awan · Y. Deng 5319

Signal peptide of cellulase
S. Yan · G. Wu 5329

Bacterial synthesis of D-amino acids
A.D. Radkov · L.A. Moe 5363

Microbial management of arthropod pests of tea: current state and prospects
S. Roy · N. Muraliedharan 5375

Atmospheric and room temperature plasma (ARTP) as a new powerful mutagenesis tool
X. Zhang · X.-F. Zhang · H.-P. Li · L.-Y. Wang · C. Zhang · X.-H. Xing · C.-Y. Bao 5387

Perspectives for the biotechnological production of ethyl acetate by yeasts
C. Löser · T. Urit · T. Bley 5397

Biotechnological products and process engineering

Effect of sodium butyrate on the assembly, charge variants, and galactosylation of antibody produced in recombinant Chinese hamster ovary cells
J.K. Hong · S.M. Lee · K.-Y. Kim · G.M. Lee 5417

Managing the cultivation and processing of microalgae to prolong storage in water-in-oil emulsions
L. Fernández · Y.-S. Cheng · H. Scher · J.S. VanderGheynst 5427

A plant malonyl-CoA synthetase enhances lipid content and polyketide yield in yeast cells
Y. Wang · H. Chen · O. Yu 5435

Process characterization and influence of alternative carbon sources and carbon-to-nitrogen ratio on organic acid production by *Aspergillus oryzae* DSM1863
K. Ochsenreither · C. Fischer · A. Neumann · C. Syltatk 5449

Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(HB-co-HHx)) from butyrate using engineered *Ralstonia eutropha*
J.-M. Jeon · C.J. Brigham · Y.-H. Kim · H.-J. Kim · D.-H. Yi · H. Kim · C. Rha · A.J. Sinskey · Y.-H. Yang 5461

Biotechnologically relevant enzymes and proteins

***Escherichia coli kduD* encodes an oxidoreductase that converts both sugar and steroid substrates**
A. Tubeleviciute · M.G. Teese · J. Jose 5471

High-level expression, purification and characterisation of porcine β -defensin 2 in *Pichia pastoris* and its potential as a cost-efficient growth promoter in porcine feed
Z. Peng · A. Wang · Q. Feng · Z. Wang · I.V. Ivanova · X. He · B. Zhang · W. Song 5487

High-level expression, purification, and characterization of bifunctional ScFv-9R fusion protein
X. Zhang · J. Xie · Y. Sun · H. Xu · T. Du · Z. Liu · J. Chen · Z. Zheng · K. Liu · J. Zhang · M. Kan · X. Li · Y. Xiao 5499

Enzymatic synthesis of model substrates recognized by glucuronoyl esterases from *Podospora anserina* and *Myceliophthora thermophila*
C. Katsimpouras · A. Bénarouche · D. Navarro · M. Karpusas · M. Dimarogona · J.-G. Berrin · P. Christakopoulos · E. Topakas 5507

The GPI-anchored protein Ecm33 is vital for conidiation, cell wall integrity, and multi-stress tolerance of two filamentous entomopathogens but not for virulence
Y. Chen · J. Zhu · S.-H. Ying · M.-G. Feng 5517

Applied genetics and molecular biotechnology

***Aspergillus niger* RhaR, a regulator involved in L-rhamnose release and catabolism**
B.S. Gruben · M. Zhou · A. Wiebenga · J. Ballering · K.M. Overkamp · P.J. Punt · R.P. de Vries 5531

***VaCPK20* gene overexpression significantly increased resveratrol content and expression of stilbene synthase genes in cell cultures of *Vitis amurensis* Rupr**
O.A. Aleynova-Shumakova · A.S. Dubrovina · A.Y. Manyakhin · Y.A. Karetin · K.V. Kiselev 5541

A disk-diffusion-based target identification platform for antibacterials (TIPA): an inducible assay for profiling MOAs of antibacterial compounds
I. Silva · L.J. Real · M.S. Ward · H.H. Xu 5551

Over-expression of native *Saccharomyces cerevisiae* exocytic SNARE genes increased heterologous cellulase secretion
J.H.D. Van Zyl · R. Den Haan · W.H. Van Zyl 5567

Use of a universal hydroxymethylbilane synthase (HMBS)-based PCR as an endogenous internal control and to enable typing of mammalian DNAs
L. Wei · P. Kelly · J. Zhang · Y. Yang · X. Zheng · J. Tao · Z. Zhang · C. Wang 5579

(Continued on inside front cover)

Applied and Microbiology Biotechnology

(Contents continued)

Genomics, transcriptomics, proteomics

Comparative transcriptomic analysis of the heat stress response in the filamentous fungus *Metarhizium anisopliae* using RNA-Seq
Z.-X. Wang · X.-Z. Zhou · H.-M. Meng · Y.-J. Liu · Q. Zhou · B. Huang 5589

Enhanced aerobic granulation, stabilization, and nitrification in a continuous-flow bioreactor by inoculating biofilms
Y. Yang · D. Zhou · Z. Xu · A. Li · H. Gao · D. Hou 5737

Role of primary substrate composition on microbial community structure and function and trace organic chemical attenuation in managed aquifer recharge systems
D. Li · M. Alidina · J.E. Drewes 5747

Applied microbial and cell physiology

Rapid adaptation of *Rhodococcus erythropolis* cells to salt stress by synthesizing polyunsaturated fatty acids
C.C.C.R. de Carvalho · M.P.C. Marques · N. Hachicho · H.J. Heipieper 5599

Bbsk1, a response regulator required for conidiation, multi-stress tolerance, and virulence of *Beauveria bassiana*
Z.-L. Wang · F. Li · C. Li · M.-G. Feng 5607

Administration of *Lactobacillus salivarius* L101 or *Pediococcus pentosaceus* L105 improves acute liver injury induced by D-galactosamine in rats
L.-X. Lv · X.-J. Hu · G.-R. Qian · H. Zhang · H.-F. Lu · B.-W. Zheng · L. Jiang · L.-J. Li 5619

Engineering of *Corynebacterium glutamicum* for growth and L-lysine and lycopene production from N-acetyl-glucosamine
C. Matano · A. Uhde · J.-W. Youn · T. Maeda · L. Clermont · K. Marin · R. Krämer · V.F. Wendisch · G.M. Seibold 5633

Evaluation of MALDI-TOF mass spectrometry for identification of environmental yeasts and development of supplementary database
B.C. Agostini · L.P. Silva · C. Bloch Jr. · T.M.B. Bonfim · G.A. da Silva 5645

Bioenergy and biofuels

2,3-Butanediol production from cellobiose by engineered *Saccharomyces cerevisiae*
H. Nan · S.-O. Seo · E.J. Oh · J.-H. Seo · J.H.D. Cate · Y.-S. Jin 5757

Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass
Y. Shang · R. Su · R. Huang · Y. Yang · W. Qi · Q. Li · Z. He 5765

Ionic liquid and deep eutectic solvent-activated CelA2 variants generated by directed evolution
C. Lehmann · M. Bocola · W.R. Streitz · R. Martinez · U. Schwaneberg 5775

Retraction Note

Retraction Note: Strategies for efficient production of heterologous proteins in *Escherichia coli*
S. Jana · J.K. Deb 5787

Methods and protocols

Improvement of DGGE analysis by modifications of PCR protocols for analysis of microbial community members with low abundance
Y.-F. Wang · F.-Q. Zhang · J.-D. Gu 5655

An AIL/IL-based liquid/liquid extraction system for the purification of His-tagged proteins
W. Xu · H. Cao · G. Ren · H. Xie · J. Huang · S. Li 5665

Affinity purification of recombinant proteins using a novel silica-binding peptide as a fusion tag
M.A.A. Abdelhamid · K. Motomura · T. Ikeda · T. Ishida · R. Hirota · A. Kuroda 5677

Occurrence and diversity of nitrite-dependent anaerobic methane oxidation bacteria in the sediments of the South China Sea revealed by amplification of both 16S rRNA and *pmoA* genes
J. Chen · Z.-C. Zhou · J.-D. Gu 5685

Environmental biotechnology

Depth-related changes of sediment ammonia-oxidizing microorganisms in a high-altitude freshwater wetland
Y. Liu · J. Zhang · X. Zhang · S. Xie 5697

Metagenomic analysis of sludge from full-scale anaerobic digesters operated in municipal wastewater treatment plants
Y. Yang · K. Yu · Y. Xia · F.T.K. Lau · D.T.W. Tang · W.C. Fung · H.H.P. Fang · T. Zhang 5709

Isolation and differentiation of methanogenic *Archaea* from mesophilic corn-fed on-farm biogas plants with special emphasis on the genus *Methanobacterium*
R. Stantschke · J. Kuever · A. Rabenstein · K. Seyfarth · S. Dröge · H. König 5719

Forthcoming articles in print

published online and citable with the DOI (Digital Object Identifier) can be found at springerlink.com

Abstracted/Indexed in Science Citation Index, Science Citation Index Expanded (SciSearch), Journal Citation Reports/Science Edition, PubMed/Medline, SCOPUS, EMBASE, Chemical Abstracts Service (CAS), Google Scholar, EBSCO, CSA, ProQuest, CAB International, Abstracts in Anthropology, Academic OneFile, Academic Search, AGRICOLA, ASFA, Biological Abstracts, BIOSIS, CAB Abstracts, CEABA-VitB, ChemWeb, CSA Environmental Sciences, Current Contents/ Agriculture, Biology & Environmental Sciences, Current Contents/ Life Sciences, EI-Compendex, Elsevier Biobase, Environment Index, Food Science and Technology Abstracts, Gale, Geobase, GeoRef, Global Health, Health Reference Center Academic, INIS Atomindex, OCLC, PASCAL, Referativnyi Zhurnal, SCImago, Summon by Serial Solutions, Vitis - Viticulture and Enology Abstracts

Applied Microbiology and Biotechnology

Aims and Scope

The journal publishes two categories of articles: (i) Original Papers (*preferred length of 6–8 printed pages*), (ii) Mini-Reviews (*preferred length of 5–6 printed pages*).

Mini-Reviews are short reviews critically summarizing the current state of and providing perspectives on biotechnological processes, products, microbial genomes, methods and equipment. Papers should deal with the following aspects of applied microbiology and biotechnology:

- **Biotechnological products and process engineering**
Established and in pipe technologies of biotechnological processes using prokaryotic or eukaryotic cells or enzymes and other proteins for production, conversion, degradation and detection of substances including bioreactor design.
- **Biotechnologically relevant enzymes and proteins**
Characterization of hitherto undescribed, biotechnologically relevant enzymes and proteins or of known proteins with novel properties and purification of proteins on a technical scale.
- **Bioenergy and biofuels**
Biotechnological processes for production of fuels, combustible chemicals and other forms of energy.
- **Applied genetics and molecular biotechnology**
New vectors and methods for genetic transformation and all aspects of strain improvement employing recombinant DNA technology including metabolic engineering and enzyme engineering.
- **Genomics, transcriptomics, proteomics**
Characterization and analyses of genomes, transcriptomes and proteomes.
- **Applied microbial and cell physiology**
Physiological studies on biosynthesis, catabolism and biotransformation of biotechnologically relevant compounds.
- **Environmental biotechnology**
All aspects of environmental processes using microorganisms and biodegradation of hazardous chemicals.
- **Methods and protocols**
Novel methods and techniques or significant modifications of already described methods or

new combinations of methods yielding significant improvements. Their novelty and reliability must be concisely presented.

Copyright Information

For Authors

As soon as an article is accepted for publication, authors will be requested to assign copyright of the article (or to grant exclusive publication and dissemination rights) to the publisher (respective the owner if other than Springer). This will ensure the widest possible protection and dissemination of information under copyright laws.

More information about copyright regulations for this journal is available at www.springer.com/253

For Readers

While the advice and information in this journal is believed to be true and accurate at the date of its publication, neither the authors, the editors, nor the publisher can accept any legal responsibility for any errors or omissions that may have been made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

All articles published in this journal are protected by copyright, which covers the exclusive rights to reproduce and distribute the article (e.g., as offprints), as well as all translation rights. No material published in this journal may be reproduced photographically or stored on microfilm, in electronic data bases, on video disks, etc., without first obtaining written permission from the publisher (respective the copyright owner if other than Springer). The use of general descriptive names, trade names, trademarks, etc., in this publication, even if not specifically identified, does not imply that these names are not protected by the relevant laws and regulations.

Springer has partnered with Copyright Clearance Center's RightsLink service to offer a variety of options for reusing Springer content. For permission to reuse our content please locate the material that you wish to use on SpringerLink.com or on SpringerImages.com and click on the permissions link or go to copyright.com and enter the title of the publication that you wish to use. For assistance in placing a permission request, Copyright Clearance Center can be connected directly via phone: +1-855-239-3415, fax: +1-978-646-8600, or e-mail: info@copyright.com.

© Springer-Verlag Berlin Heidelberg 2014

V.i.S.d.P. Alexander Steinbüchel (Editor-in-Chief)

Journal Website

www.springer.com/00253
Electronic edition:
link.springer.com/journal/00253

Subscription Information

Applied Microbiology and Biotechnology is published 24 times a year. Volume 98 (24 issues) will be published in 2014.

ISSN print edition 0175-7598
ISSN electronic edition 1432-0614

For information on subscription rates please contact Springer Customer Service Center:

The Americas (North, South, Central America and the Caribbean)

Springer Journal Fulfillment
233 Spring Street, New York
NY 10013-1578, USA
Tel.: 800-SPRINGER (777-4643);
212-460-1500 (outside North America)
journals-ny@springer.com;
servicio-ny@springer.com
(Central and South America)

Outside the Americas

Springer Customer Service Center GmbH
Haberstr. 7, 69126 Heidelberg, Germany
Tel.: +49-6221-345-4303
subscriptions@springer.com

Advertisements

E-mail contact: advertising@springer.com or
anzeigen@springer.com (Germany)

Disclaimer

Springer publishes advertisements in this journal in reliance upon the responsibility of the advertiser to comply with all legal requirements relating to the marketing and sale of products or services advertised. Springer and the editors are not responsible for claims made in the advertisements published in the journal. The appearance of advertisements in Springer publications does not constitute endorsement, implied or intended, of the product advertised or the claims made for it by the advertiser.

Office of Publication

Springer-Verlag GmbH, Tiergartenstraße 17,
69121 Heidelberg, Germany

Springer is part of
Springer Science+Business Media

MINI-REVIEW

Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production

Mudassar Ahmad · Melanie Hirz · Harald Pichler ·
Helmut Schwab

Received: 27 January 2014 / Revised: 25 March 2014 / Accepted: 26 March 2014 / Published online: 18 April 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract *Pichia pastoris* is an established protein expression host mainly applied for the production of biopharmaceuticals and industrial enzymes. This methylotrophic yeast is a distinguished production system for its growth to very high cell densities, for the available strong and tightly regulated promoters, and for the options to produce gram amounts of recombinant protein per litre of culture both intracellularly and in secretory fashion. However, not every protein of interest is produced in or secreted by *P. pastoris* to such high titres. Frequently, protein yields are clearly lower, particularly if complex proteins are expressed that are hetero-oligomers, membrane-attached or prone to proteolytic degradation. The last few years have been particularly fruitful because of numerous activities in improving the expression of such complex proteins with a focus on either protein engineering or on engineering the protein expression host *P. pastoris*. This review refers to established tools in protein expression in *P. pastoris* and highlights novel developments in the areas of expression vector design, host strain engineering and screening for high-level expression strains. Breakthroughs in membrane protein expression are discussed alongside numerous commercial applications of *P. pastoris* derived proteins.

Keywords Yeast · *Pichia pastoris* · Protein expression · Protein secretion · Protease-deficient strains · Chaperone

Mudassar Ahmad and Melanie Hirz contributed equally to this work.

M. Ahmad · M. Hirz · H. Pichler · H. Schwab (✉)
Institute of Molecular Biotechnology, Graz University of
Technology, Petersgasse 14/5, 8010 Graz, Austria
e-mail: helmut.schwab@tugraz.at

H. Pichler · H. Schwab
Austrian Centre of Industrial Biotechnology (ACIB), Petersgasse 14,
8010 Graz, Austria

Introduction

The methylotrophic yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris*, has become a substantial workhorse for biotechnology, especially for heterologous protein production (Kurtzman 2009). It was introduced more than 40 years ago by Phillips Petroleum for commercial production of single cell protein (SCP) as animal feed additive based on a high cell density fermentation process utilizing methanol as carbon source. However, the oil crisis in 1973 increased the price for methanol drastically and made SCP production uneconomical. In the 1980s, *P. pastoris* was developed as a heterologous protein expression system using the strong and tightly regulated *AOX1* promoter (Cregg et al. 1985). In combination with the already developed fermentation process for SCP production, the *AOX1* promoter provided exceptionally high levels of heterologous proteins. One of the first large-scale industrial production processes established in the 1990s was the production of the plant-derived enzyme hydroxynitrile lyase at >20 g of recombinant protein per litre of culture volume (Hasslacher et al. 1997). This enzyme is used as biocatalyst for the production of enantiopure *m*-phenoxybenzaldehyde cyanohydrin — a building block of synthetic pyrethroids — on the multi-ton scale.

Through a far-sighted decision this expression system, initially patented by Phillips Petroleum, was made available to the scientific community for research purposes. A major breakthrough was the publication of detailed genome sequences of the original SCP production strain CBS7435 (Küberl et al. 2011), the first host strain developed for heterologous protein expression GS115 (De Schutter et al. 2009), as well as of the related *P. pastoris* DSMZ 70382 strain (Mattanovich et al. 2009b). Equally important breakthroughs for the commercial application of the *P. pastoris* cell factory were the Food and Drug Administration (FDA) GRAS (generally recognized as safe) status for a protein used in animal

feed, phospholipase C (Ciofalo et al. 2006), and the FDA approval of a recombinant biopharmaceutical product, Kalbitor[®], a kallikrein inhibitor (Thompson 2010).

The classical *P. pastoris* expression system has been extensively reviewed over the years (Cereghino and Cregg 2000; Daly and Hearn 2005; Gasser et al. 2013; Jin et al. 2006; Macauley-Patrick et al. 2005). In this review, we focus on recent developments for heterologous protein production and describe examples for the commercial use of this expression system. In the first chapter, we refer to the established basic vector systems and elaborate on developments thereof with an emphasis on newly developed promoter systems. Herein, also some aspects of secretion will be summarized. The second part is devoted to the most recent developments regarding host strain development. As a specific novelty, a new platform based on the CBS7435 strain is described, for which patent protection has ceased and no specific material rights are pending. In the third chapter, we describe specific strategies for obtaining high-level expression strains and summarize important applications of *P. pastoris* for production of biopharmaceuticals, membrane proteins and industrial proteins. The last section provides an outlook on future perspectives covering recent progress in molecular and cell biology of *P. pastoris* and possibilities for implementing new strategies in expression strain development.

Basic systems for cloning and expression in *P. pastoris*

When devising strategies for cloning and expression of heterologous proteins in *P. pastoris* some points need to be considered from the start, that is, the choice of promoter–terminator combinations, suitable selection markers and application of vector systems for either intracellular or secreted expression including selection of proper secretion signals (Fig. 1). The choice of the proper expression vector and complementary host strain are a most important prerequisite for successful recombinant protein expression.

Promoters

The use of tightly regulated promoters such as the alcohol oxidase (*AOX1*) promoter holds advantages for overexpression of proteins. By uncoupling the growth from the production phase, biomass is accumulated prior to protein expression. Therefore, cells are not stressed by the accumulation of recombinant protein during growth phase, and even the production of proteins that are toxic to *P. pastoris* is possible. Furthermore, it may be desirable to co-express helper proteins like chaperones at defined time points, for example, before the actual target protein is formed. On the other hand, use of constitutive promoters may ease process handling. Constitutive promoters are usually also applied to express

selection markers. Metabolic pathway engineering strategies might further take advantage of fine-tuned constitutive promoters to ensure a controlled flux of metabolites. An extensive summary of promoters used for heterologous expression in *P. pastoris* has recently been published by Vogl and Glieder (2013). An overview of broadly used and extensively studied as well as recently examined promoters is given in Table 1.

Inducible promoters

The tightly regulated *AOX1* promoter (P_{AOX1}), which was first employed for heterologous gene expression by Tschopp et al. (1987a), is still the most commonly used promoter (Lünsdorf et al. 2011; Sigoillot et al. 2012; Yu et al. 2013). P_{AOX1} is strongly repressed when *P. pastoris* is grown on glucose, glycerol or ethanol (Inan and Meagher 2001). Upon depletion of these carbon sources, the promoter is de-repressed, but is fully induced only upon addition of methanol. Several studies have identified multiple regulatory elements in the P_{AOX1} sequence (Hartner et al. 2008; Kranthi et al. 2006, 2009; Ohi et al. 1994; Parua et al. 2012; Staley et al. 2012; Xuan et al. 2009). Positively and negatively acting elements have been described (Kumar and Rangarajan 2012; Lin-Cereghino et al. 2006; Polupanov et al. 2012), but the molecular details of P_{AOX1} regulation are still not completely elucidated.

Methanol is a highly flammable and hazardous substance and, therefore, undesirable for large-scale fermentations. Alternative inducible promoters or P_{AOX1} variants, which can be induced without methanol but still reach high expression levels, are desired. A recently published patent application describes such a method, wherein expression is controlled by methanol-inducible promoters, such as *AOX1*, methanol oxidase (*MOX*) or formate dehydrogenase (*FMDH*), without the addition of methanol (Takagi et al. 2008). This was achieved by constitutively co-expressing the positively acting transcription factor Prm1p from either of the *GAP*, *TEF* or *PGK* promoters. The relative activity of a phytase reporter protein was 3-fold increased without addition of methanol as compared to a control strain with *PRM1* under its native promoter. However, phytase expression levels were not compared for standard methanol induction and constitutive Prm1p expression conditions. Hartner et al. have constructed a synthetic *AOX1* promoter library by deleting or duplicating transcription factor binding sites for fine-tuned expression in *P. pastoris* (Hartner et al. 2008). Using EGFP as reporter, some promoter variants were found to confer even higher expression levels than the native P_{AOX1} spanning a range between 6 % and 160 % of the native promoter activity. These P_{AOX1} variants have also proven to behave similarly when industrially relevant enzymes such as horseradish peroxidase and hydroxynitrile lyases were expressed.

Numerous further controllable promoters are currently being investigated for their ability to promote high-level

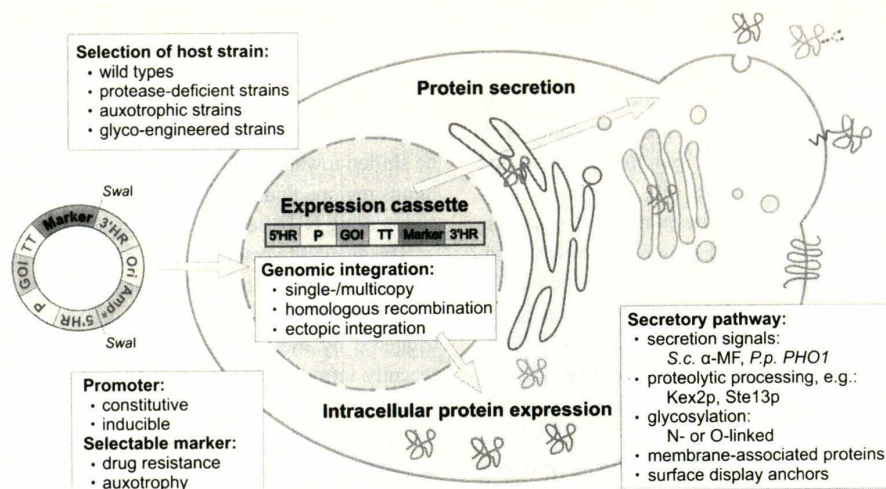


Fig. 1 General considerations for heterologous gene expression in *P. pastoris*. Expression plasmids harbouring the gene(s) of interest (*GOI*) are linearized prior to transformation. Selectable markers (e.g., *Amp^R*) and origin of replication (*Ori*) are required for plasmid propagation in *E. coli*. The expression level of the protein of interest may depend on (i) the chromosomal integration locus, which is targeted by the 5' and

3' homologous regions (5'HR and 3'HR), and (ii) on the gene copy number. A representative promoter (*P*) and transcription terminator (*TT*) pair are shown. Proper signal sequences will guide recombinant protein for intracellular or secretory expression, and will govern membrane integration or membrane anchoring

expression (Table 1). For example, a recently published patent application describes the use of three novel inducible promoters from *P. pastoris*, *ADHI* (alcohol dehydrogenase), *GUT1* (glycerol kinase) and *ENO1* (enolase), showing

interesting regulatory features (Cregg and Tolstorukov 2012). However, due to a lack of absolute expression values the performance of these novel promoters cannot be compared to the widely used *AOX1* and *GAP* promoters.

Table 1 The most prominently used and very recently established promoters for heterologous expression in *P. pastoris*

Inducible	Corresponding gene	Regulation	Reference
<i>AOX1</i>	Alcohol oxidase 1	Inducible with MeOH	(Tschopp et al. 1987a)
<i>DAS</i>	Dihydroxyacetone synthase	Inducible with MeOH	(Ellis et al. 1985; Tschopp et al. 1987a)
<i>FLD1</i>	Formaldehyde dehydrogenase 1	Inducible with MeOH or methylamine	(Shen et al. 1998)
<i>ICL1</i>	Isocitrate lyase	Repressed by glucose, induction in absence of glucose/by addition of ethanol	(Menendez et al. 2003)
<i>PHO89</i>	Putative Na ⁺ /phosphate symporter	Induction upon phosphate starvation	(Ahn et al. 2009)
<i>THI11</i>	Thiamine biosynthesis gene	Repressed by thiamin	(Stadlmayr et al. 2010)
<i>ADHI</i>	Alcohol dehydrogenase	Repressed on glucose and methanol, induced on glycerol and ethanol	(Cregg and Tolstorukov 2012)
<i>ENO1</i>	Enolase	Repressed on glucose, methanol and ethanol, induced on glycerol	(Cregg and Tolstorukov 2012)
<i>GUT1</i>	Glycerol kinase	Repressed on methanol, induced on glucose, glycerol and ethanol	(Cregg and Tolstorukov 2012)
Constitutive	Corresponding gene	Regulation	Reference
<i>GAP</i>	Glyceraldehyde-3-P dehydrogenase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(Waterham et al. 1997)
<i>TEF1</i>	Translation elongation factor 1	Constitutive expression on glycerol and glucose	(Ahn et al. 2007)
<i>PGK1</i>	3-Phosphoglycerate kinase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(de Almeida et al. 2005)
<i>GCW14</i>	Potential glycosyl phosphatidyl inositol (GPI)-anchored protein	Constitutive expression on glycerol, glucose and methanol	(Liang et al. 2013b)
<i>G1</i>	High affinity glucose transporter	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)
<i>G6</i>	Putative aldehyde dehydrogenase	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)

Constitutive promoters

Constitutive expression eases process handling, omits the use of potentially hazardous inducers and provides continuous transcription of the gene of interest. For this purpose, the glyceraldehyde-3-phosphate promoter (P_{GAP}) is commonly used, which — on glucose — reaches almost the same expression levels as methanol-induced P_{AOX1} (Waterham et al. 1997). Expression levels from P_{GAP} drop to about one half on glycerol and to one third when cells are grown on methanol (Cereghino and Cregg 2000). Alternative constitutive promoters and promoter variants have been described recently (Table 1). The constitutive P_{GCW14} promoter, for example, was described to be a stronger promoter than the GAP and $TEF1$ promoters, which was assessed by secretory expression of EGFP (Liang et al. 2013b). It was found that EGFP expression from P_{GCW14} yielded in a 10-fold increase compared to P_{GAP} driven expression when cells were cultivated on glycerol or methanol, and a 5-fold increase on glucose.

A recent DNA microarray study identified novel promoters that are repressed on glycerol, but are being induced upon shift to glucose-limited media (Prielhofer et al. 2013). Supposedly, the most interesting promoters discovered by this approach control expression of a high-affinity glucose transporter, $HGT1$, and of a putative aldehyde dehydrogenase. The former promoter was reported to drive EGFP expression to even higher levels than could be reached with P_{GAP} . In glycerol fed-batch fermenter cultures, human serum album was expressed from the novel promoter to a 230 % increase in specific product yield as compared to P_{GAP} driven expression.

In some cases, it is desired that expression levels can be fine-tuned in order to (1) co-express accessory proteins facilitating recombinant protein expression and secretion or (2) provide protein post-translational modifications as well as to (3) engineer whole metabolic pathways consisting of a cascade of different enzymatic steps. For such applications, a library of GAP promoter variants with relative strengths ranging from 0.6 % to 16.9-fold of the wild type promoter activity was developed and tested using three different reporter proteins, yEGFP, β -galactosidase and methionine acetyltransferase (Qin et al. 2011).

Vectors

The standard setup of vectors is a bi-functional system enabling replication in *E. coli* and maintenance in *P. pastoris* using as selection markers either auxotrophy markers (e.g., $HIS4$, $MET2$, $ADE1$, $ARG4$, $URA3$, $URA5$, $GUT1$) or genes conferring resistance to drugs such as Zeocin™, geneticin (G418) and blasticidin S. Although there are some reports of using episomal plasmids for heterologous protein expression or for the screening of mutant libraries in *P. pastoris* (Lee et al. 2005; Uchima and Arioka 2012), stable integration into the

host genome is the most preferred method. Unlike in *Saccharomyces cerevisiae*, where homologous recombination (HR) predominates, non-homologous end-joining (NHEJ) is a frequent process in *P. pastoris*. The ratio of NHEJ and HR can be shifted towards HR by elongating the length of the homologous regions flanking the actual expression cassettes and by suppressing NHEJ efficiency (Näätäsaari et al. 2012).

The standard vector systems for intracellular and secretory expression provided by Life Technologies (Carlsbad, CA, USA) include constitutive (P_{GAP}) and inducible promoters triggered by methanol or methylamine (P_{AOX1} , P_{FLD}). The recently introduced PichiaPink™ expression kit for intracellular or secreted expression enables easy selection of multicopy integration clones by differences in colour formation based on *ade2* knockout strains and truncated *ADE2* promoters of varying strengths in front of the *ADE2* marker gene (Du et al. 2012; Nett 2010).

Additionally, BioGrammatics (Carlsbad, CA, USA) holds licences for selling standard *P. pastoris* expression vectors and strains and also provides GlycoSwitch® vectors for humanized glycosylation of target proteins (Table 2). Several vectors for disruption of *OCH1* and expression of different glycosidases or glycosyltransferases are available to achieve mammalian-type N-glycan structures in *P. pastoris*. These vectors harbour, for example, the human GlcNAc transferase I, the mannosidase II from rat, or the human galactosyl transferase I. A detailed protocol for humanizing the glycosylation pattern using the GlycoSwitch® vectors is provided (Jacobs et al. 2009).

James Cregg's laboratory at the Keck Graduate Institute, Claremont, CA, USA, has developed a set of plasmids for protein secretion and intracellular expression in *P. pastoris* containing the strong *AOX1* promoter. These vectors are based on different auxotrophy markers, such as *ARG4*, *ADE1*, *URA3* and *HIS4*, for selection necessitating the use of the appropriate host strains (see section "Host strain development"). The vectors contain restriction sites for linearization within the marker genes to target the expression cassettes to the desired locus as well as for multicopy integration (Lin-Cereghino et al. 2001). Moreover, a set of integration vectors for sequential disruption of *ARG1*, *ARG2*, *ARG3*, *HIS1*, *HIS2*, *HIS5* and *HIS6* in *P. pastoris* was applied to provide the host strains for engineering the protein glycosylation pathway (Nett et al. 2005).

The Institute of Molecular Biotechnology, Graz University of Technology, Austria, provides vectors and strains to the *P. pastoris* community through the so-called 'Pichia Pool'. The pPp plasmids described by Näätäsaari et al. (2012) comprise vectors containing the GAP or $AOX1$ promoters and, for secretory expression, the *S. cerevisiae* α -mating factor (α -MF) secretion signal. The antibiotic selection marker cassettes were placed under the control of *ADHI* or *ILV5* promoters in the pPpB1 and pPpT4 vectors, respectively. It is

Table 2 Commercial vector systems

Supplier	Promoter	Signal sequences	Selection in yeast	Selection in bacteria	Comments
Life Technologies™	<i>AOX1</i> , <i>FLD1</i> , <i>GAP</i>	<i>S. cerevisiae</i> α -MF; <i>P. pastoris</i> <i>PHO1</i>	Blasticidin, G418, Zeocin™, <i>HIS4</i>	Zeocin™, Ampicillin, Blasticidin	c-myc epitope, V5 epitope, C-terminal 6× His-tag available for detection/purification
Life Technologies –PichiaPink™	<i>AOX1</i>	α -MF; set of eight different signal sequences – not ready to use ^a	<i>ADE2</i>	Ampicillin	Low- and high-copy vectors available, <i>TRP2</i> sequence for targeting
BioGrammatics	<i>AOX1</i>	α -MF	Zeocin™, G418, Nourseothricin	Ampicillin	Intracellular or secreted expression
BioGrammatics – GlycoSwitch®	<i>GAP</i>	–	Zeocin™, G418, Hygromycin, <i>HIS4</i> , Nourseothricin	Zeocin™, Ampicillin, Kanamycin, Nourseothricin	Human GlcNAc transferase I, rat Mannosidase II, human Gal transferase I
DNA2.0	<i>AOX1</i>	Ten different signal sequences – ready to use ^b	Zeocin™, G418	Zeocin™, Ampicillin	Intracellular or secreted

^a The different secretion signals have to be cloned into the vector by a three-way ligation step

^b The α -MF secretion signal is provided once with Kex2p (KR) and Ste13p cleavage sites (EAEA), once lacking EA repeats, and once as truncated version (pre-region only)

described that the *pPpT4*-based vectors usually lead to lower gene copies in the cell as compared to the *pPpB1*-based vectors.

Further vectors based on either the *GAP* or the *AOX1* promoter and a series of strains have recently been added to this pool, both for intracellular and secretory protein expression (M. Ahmad, unpublished results). For intracellular expression, cloning of the target genes is accomplished by using *EcoRI* and *NotI*, whereby the Kozak consensus sequence has to be restored for efficient translation initiation (Fig. 2a). A special characteristic of these vectors is that the *EcoRI* site has been introduced by a single point mutation directly into the *AOX1* promoter sequence without changing the promoter activity. Thereby, the gene of interest may be fused to the promoter without having additional nucleotides between the promoter and the start codon. Another advantage is the use of the short *ARG4* promoter for the expression of the selection markers. The weaker *ARG4* promoter used for selection marker cassettes enables selection at lower concentrations of Zeocin™ (i.e., 25 instead of 100 μ g/ml) without obtaining false-positive clones. For secretory expression governed by the *S. cerevisiae* α -MF signal sequence, *XhoI* and/or *NotI* sites are used for cloning the genes of interest (Fig. 2b).

Aspects of secretory expression

One of the main advantages of using *P. pastoris* as a protein production host is its ability to secrete high titres of properly folded, post-translationally processed and active recombinant proteins into the culture media. As a rule of thumb, proteins secreted in their native hosts will also be secreted in *P. pastoris*. However, there are also some reports of successful

secretion of typically intracellular proteins such as GFP or human catalase (Eiden-Plach et al. 2004; Shi et al. 2007). The most commonly employed secretion signals in *P. pastoris* are derived from *S. cerevisiae* α -MF, *S. cerevisiae* invertase (*SUC2*) and the *P. pastoris* endogenous acid phosphatase (*PHO1*) (Daly and Hearn 2005). As listed in Table 2, commercial kits also provide vectors with different secretion signals, which allows for screening of the best-suited signal sequence.

The α -MF signal sequence is composed of a pre- and pro-region and has proven to be most effective in directing protein through the secretory pathway in *P. pastoris*. The pre-region is responsible for directing the nascent protein post-translationally into the endoplasmic reticulum (ER) and is cleaved off subsequently by signal peptidase (Waters et al. 1988). The pro-region is thought to play a role in transferring the protein from ER to Golgi compartment and is finally cleaved at the dibasic KR site by the endo-protease Kex2p (Julius et al. 1984). The two EA repeats are subsequently trimmed by the *STE13* gene product (Brake et al. 1984). One of the common problems encountered while using the α -MF secretion signal is non-homogeneity of the N-termini of the recombinant proteins due to incomplete *STE13* processing. Constructs without the EA repeats may enhance homogeneity at the N termini of recombinant proteins. However, the removal of these sequences may affect protein yield. While no reports on enhanced co-expression of *STE13* are available, co-overexpression of *HAC1*, a transcription factor in the unfolded protein response (UPR) pathway, with the membrane protein adenosine A2 receptor had a positive effect on proper processing of the α -MF signal sequence (Guerfal

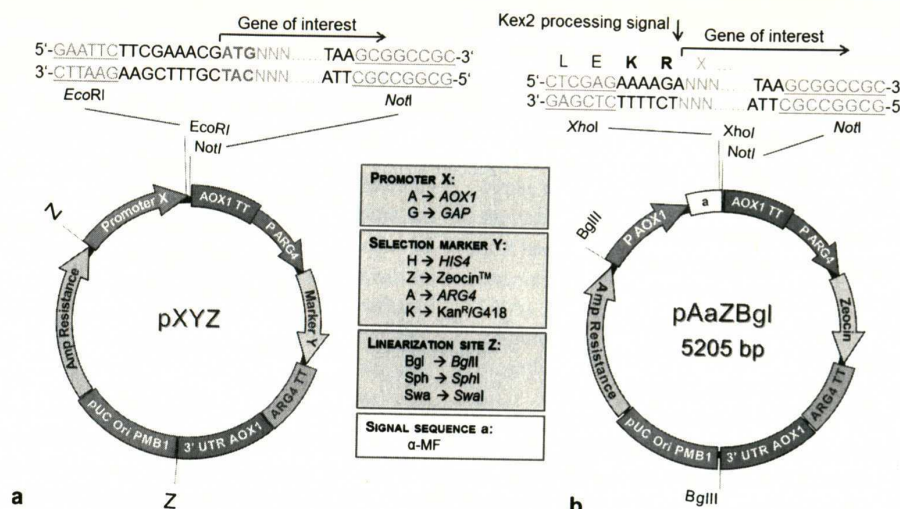


Fig. 2 Novel 'Pichia Pool' plasmid sets for intracellular and secretory expression. **a** General features of pXYZ vector for intracellular expression. Letters refer to the choice of promoters (X), selection markers (Y), and restriction enzymes (Z) for linearization. Available elements are shown in boxes. The vector backbone harbours an ampicillin resistance marker and origin of replication for maintenance of the plasmid in *E. coli*. The GOI is *EcoRI*–*NotI* cloned directly after the promoter of choice. The Kozak consensus sequence for yeast (i.e., CGAAACG), should be restored between the *EcoRI* cloning site and the start codon of the GOI in order to achieve optimal translation. In addition, sequence variation

within this region will allow fine-tuning translation initiation efficiency. Expression in *P. pastoris* is driven either by the methanol inducible *AOX1* or the constitutive *GAP* promoter. Positive clones can be selected for by antibiotic resistance (i.e., to Zeocin™ or geneticin sulphate) or by selection for His or Arg prototrophy. Selection marker expression is uniformly driven by the *ARG4* promoter–terminator pair. **b** Plasmid pAaZBgl from 'Pichia Pool' is shown as an example of a vector made for secretory expression encoding *S. cerevisiae* α-MF signal sequence in front of the GOI cloning site. The Kex2 processing site AAAAGA should be restored between the *XhoI* cloning site and the fusion point of the GOI

et al. 2010). Recently, Yang et al. (2013) reported enhanced secretory protein production by optimizing the amino acid residues at the Kex2 P1' site.

Multiple strategies have been followed to enhance the secretory potential of the α-MF signal sequence including codon optimization (Kjeldsen et al. 1998), directed evolution (Rakestraw et al. 2009), insertion of spacers and deletion mutagenesis (Lin-Cereghino et al. 2013). Directed evolution of the α-MF signal sequence in *S. cerevisiae* resulted in up to 16-fold enhanced full-length IgG₁ secretion as compared to the wild type. Furthermore, when this improved leader sequence was combined with strain engineering strategies comprising *PDI* overexpression and elimination of proteins involved in vacuolar targeting, up to 180-fold enhanced secretion of the reporter protein was observed (Rakestraw et al. 2009). Deletion mutagenesis based on a predicted structure model of α-MF signal peptide resulted in 50 % increased secretion of horseradish peroxidase and *C. antarctica* lipase B (CALB) in *P. pastoris* (Lin-Cereghino et al. 2013). It appears that decreasing the hydrophobicity of the leader sequence by deleting hydrophobic residues or substituting them with more polar or charged residues increased the flexibility of the α-MF signal sequence structure, which enhanced the overall secretory capacity of the pro-region. Alternative signal sequences used to direct protein secretion and their features and applications are summarized in Table 3.

Beyond the choice of the secretion signals there are several other factors that govern efficient protein secretion. The newly synthesized proteins are translocated co- or post-translationally into the ER lumen through the Sec61p translocon. Then, proteins may undergo one or several post-translational modifications, folding into the native state, disulphide-bond formation, glycosylation and membrane-anchoring. When the recombinant protein fails to fold into its native state or protein expression exceeds the folding capacity of the ER (Sha et al. 2013), unfolded proteins may start to aggregate, triggering the UPR pathway. UPR is responsible for induction of genes that are involved in protein folding. In parallel to UPR pathway, ER-associated degradation (ERAD) by the proteasome may relieve blocks in protein secretion (recently reviewed by Idiris et al. 2010 and Damasceno et al. 2012). Inappropriate mRNA structure and gene copy numbers, limits in transcription, translation and protein translocation into the ER, incomplete protein folding and inefficient protein targeting to the exterior of the cell are major bottlenecks encountered in secretory expression of heterologous proteins. Commonly used strategies to overcome such secretory bottlenecks comprise the overexpression of folding helper proteins like BiP/Kar2p, DnaJ, PDI, PPIs and Ero1p or, alternatively, overexpression of *HAC1*, a transcriptional regulator of the UPR pathway genes. Unlike in *S. cerevisiae*, Guerfal et al. (2010) reported that *HAC1* is

Table 3 Signal sequences used to secrete the protein into the extracellular space

Secretion signal	Source	Target protein(s)	Length	Reference
α -MF	<i>S.c.</i> α -mating factor	Most commonly used secretion signal in <i>P. pastoris</i>	85 aa, with or without EA repeats	(Brake et al. 1984)
PHO1	<i>P.p.</i> acid phosphatase	Mouse 5-HT5A, porcine pepsinogen,	15 aa	(Payne et al. 1995; Weiss et al. 1995; Yoshimasu et al. 2002)
SUC2	<i>S.c.</i> Invertase	Human interferon, α -amylase, α -1-antitrypsin	19 aa	(Moir and Dumais 1987; Paifer et al. 1994; Tschopp et al. 1987b)
PHA-E	Phytohemagglutinin	GNA, GFP and native protein	21 aa	(Raemaekers et al. 1999)
KILM1	K1 toxin	CM cellulase	44 aa	(Skipper et al. 1985)
pGKL	pGKL killer protein	Mouse α -amylase	20 aa	(Kato et al. 2001)
CLY and CLY-L8	C-lysozyme and syn. leucin-rich peptide	Human lysozyme	18 and 16 aa	(Oka et al. 1999)
K28 pre-pro-toxin	K28 virus toxin	Green fluorescent protein	36 aa	(Eiden-Plach et al. 2004)
Scw, Dse and Exg	<i>P.p.</i> Endogenous signal peptides	CALB and EGFP	19, 20 and 23 aa	(Liang et al. 2013a)
<i>Pp</i> Pir1	<i>P.p.</i> Pir1p	EGFP and Human α 1-antitrypsin	61 aa	(Khasa et al. 2011)
HBFI and HBFII	Hydrophobins of <i>Trichoderma reesei</i>	EGFP	16 and 15 aa	(Kottmeier et al. 2011)

constitutively expressed and spliced in *P. pastoris* under normal growth conditions, which may explain the higher titers of secreted proteins obtainable with this organism. A contradictory observation was reported by Whiteside et al. (2011). Unspliced *HAC1* mRNA was detected under normal growth conditions and splicing of *HAC1* mRNA was only detected when cells were grown in presence of dithiothreitol (DTT) to activate the UPR. It should be mentioned, though, that sometimes overexpression of folding helpers actually reduced protein secretion or did not have any effect (van der Heide et al. 2002).

Host strain development

Elucidation of full genome sequences and gene annotation were great steps toward rational strain engineering, identifying new promoters and progressing in the (systems) biology of *P. pastoris* (Küberl et al. 2011; Mattanovich et al. 2009a; De Schutter et al. 2009). Two online databases (<http://bioinformatics.psb.ugent.be/orcae/overview/Picpa> and <http://www.pichiagenome.org>) provide convenient access to genome sequences and annotations. Frequently used commercially available strains are the *his4* strain GS115, the reconstituted prototrophic strain X-33, the *aox1* knockout strains KM71 and KM71H as well as protease-deficient strains SMD1168 and SMD1168H and the *ade2* auxotrophic *PichiaPink*TM strain. Use of these strains for commercial applications, however, is restricted by patent protection and/or materials ownership policy. Strains derived from *P. pastoris* CBS7435, in contrast, are not covered by patent protection and, therefore represent an alternative for production purposes. Furthermore, the CBS7435 Mut^S strain provided by

the Graz *Pichia* Pool has the advantage of being marker-free as it was constructed using the Flp/FRT recombinase system for marker removal (Näätäsaari et al. 2012). Using the same strategy, *ade1* and *his4* knockout strains were created along with the CBS7435 *ku70* strain (CBS 12694), which is impaired in the NHEJ mechanism, thereby enhancing the efficiency of HR. A selection of most relevant strains is compiled in Table 4.

Auxotrophic strains

Several auxotrophic strains (e.g., *ade1*, *arg4*, *his4*, *ura3*, *met2*), and combinations thereof are available together with vectors harbouring the respective genes as selectable markers (Lin-Cereghino et al. 2001; Thor et al. 2005, Graz *Pichia* Pool). Auxotrophic strains have been useful for in vivo labelling of proteins, for example in the global fluorination of *Candida antarctica* lipase B (CALB) in a *P. pastoris* X-33 *aro1* strain deficient in tryptophan, tyrosine, and phenylalanine biosynthesis (Budisa et al. 2010). Fluorinated analogues of these amino acids were supplemented and incorporated into the heterologous protein, thereby, for example, prolonging CALB shelf-life but lowering its lipase activity. The proteolytic pattern of CALB was retained, though. Another example is the use of a *lys2 arg4* double knockout strain for stable isotope labelling by amino acids in cell culture (SILAC) (Austin et al. 2011).

Protease-deficient strains

Undesired proteolysis of heterologous proteins expressed in *P. pastoris* does not only lower the product yield or biological

Table 4 *P. pastoris* host strains

Strain	Genotype	Phenotype	Source
Wild-type strains			
CBS7435 (NRRL Y-11430)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
CBS704 (DSMZ 70382)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
X-33	WT	WT	Life Technologies™
Auxotrophic strains			
GS115	<i>his4</i>	His [−]	Life Technologies™
PichiaPink™ 1	<i>ade2</i>	Ade [−]	Life Technologies™
KM71	<i>his4, aox1::ARG4, arg4</i>	His [−] , Mut ^S	Life Technologies™
KM71H	<i>aox1::ARG4, arg4</i>	Mut ^S	Life Technologies™
BG09	<i>arg4::nourseo^R Δlys2::hyg^R</i>	Lys [−] , Arg [−] , Nourseothricin ^R , Hygromycin ^R	BioGrammatics
GS190	<i>arg4</i>	Arg [−]	(Cregg et al. 1998)
GS200	<i>arg4 his4</i>	His [−] , Arg [−]	(Waterham et al. 1996)
JC220	<i>ade1</i>	Ade [−]	(Cregg et al. 1998)
JC254	<i>ura3</i>	Ura [−]	(Cregg et al. 1998)
JC227	<i>ade1 arg4</i>	Ade [−] Arg [−]	(Lin-Cereghino et al. 2001)
JC300-JC308	Combinations of <i>ade1 arg4 his4 ura3</i>	Combinations of Ade [−] , Arg [−] , His [−] , Ura [−]	(Lin-Cereghino et al. 2001)
YJN165	<i>ura5</i>	Ura [−]	(Nett and Gerngross 2003)
CBS7435 <i>his4</i> ^a	<i>his4</i>	His [−]	(Näätäsaari et al. 2012)
CBS7435 Mut ^S <i>his4</i> ^a	<i>aox1, his4</i>	Mut ^S , His [−]	(Näätäsaari et al. 2012)
CBS7435 Mut ^S <i>arg4</i> ^a	<i>aox1, arg4</i>	Mut ^S , Arg [−]	(Näätäsaari et al. 2012)
CBS7435 <i>met2</i> ^a	<i>met2</i>	Met [−]	(Pp7030) ^b
CBS7435 <i>met2 arg4</i> ^a	<i>met2 arg4</i>	Met [−] Arg [−]	(Pp7031) ^b
CBS7435 <i>met2 his4</i> ^a	<i>met2 his4</i>	Met [−] His [−]	(Pp7032) ^b
CBS7435 <i>lys2</i> ^a	<i>lys2</i>	Lys [−]	(Pp7033) ^b
CBS7435 <i>lys2 arg4</i> ^a	<i>lys2 arg4</i>	Lys [−] Arg [−]	(Pp7034) ^b
CBS7435 <i>lys2 his4</i> ^a	<i>lys2 his4</i>	Lys [−] His [−]	(Pp7035) ^b
CBS7435 <i>pro3</i> ^a	<i>pro3</i>	Pro [−]	(Pp7036) ^b
CBS7435 <i>tyr1</i> ^a	<i>tyr1</i>	Tyr [−]	(Pp7037) ^b
Protease-deficient strains			
SMD1163	<i>his4 pep4 prb1</i>	His [−]	(Gleeson et al. 1998)
SMD1165	<i>his4 prb1</i>	His [−]	(Gleeson et al. 1998)
SMD1168	<i>his4 pep4::URA3 ura3</i>	His [−]	Life Technologies™
SMD1168H	<i>pep4</i>	His [−]	Life Technologies™
SMD1168 <i>kex1::SUC2</i>	<i>pep4::URA3 kex1::SUC2 his4 ura3</i>	His [−]	(Boehm et al. 1999)
PichiaPink 2-4	Combinations of <i>prb1/pep4</i>	Ade [−]	Life Technologies™
BG21	<i>sub2</i>		BioGrammatics
CBS7435 <i>prc1</i> ^a	<i>prc1</i>		(Pp6676) ^b
CBS7435 <i>sub2</i> ^a	<i>sub2</i>		(Pp6668) ^b
CBS7435 <i>sub2</i> ^a	<i>his4 pep4</i>	His [−]	(Pp6911) ^b
CBS7435 <i>prb1</i> ^a	<i>prb1</i>		(Pp6912) ^b
CBS7435 <i>his4 pep4 prb1</i>	<i>his4 pep4 prb1</i>	His [−]	(Pp7013) ^b
Glyco-engineered strains			
SuperMan ₅	<i>his4 och1::pGAPTrα1,2-mannosidase</i>	His [−] , Blastidicin ^R	BioGrammatics
	<i>och1::pGAPTrα1,2-mannosidase</i>	Blastidicin ^R	BioGrammatics
	<i>pep4 och1::pGAPTrα1,2-mannosidase</i>	Blastidicin ^R	BioGrammatics

Table 4 (continued)

Strain	Genotype	Phenotype	Source
Other strains			
GS241	<i>fld1</i>	Growth defect on methanol as sole C-source or methylamine as sole N-source	(Shen et al. 1998)
MS105	<i>his4 fld1</i>	See GS241; His [−]	(Shen et al. 1998)
MC100-3	<i>his4 arg4 aox1::ScARG4 aox2::PpHIS4</i>	Mut [−]	(Cregg et al. 1989)
CBS7435 <i>ku70</i> ^a	<i>ku70</i>	WT	(Näätsaari et al. 2012)
CBS7435 <i>ku70 his4</i> ^a	<i>ku70, his4</i>	His [−]	(Näätsaari et al. 2012)
CBS7435 <i>ku70 gut1</i>	<i>ku70, gut1</i>	Growth defect on glycerol; Zeocin ^R	(Näätsaari et al. 2012)
CBS7435 <i>ku70 ade1</i>	<i>ku70, ade1</i>	Ade [−] , Zeocin ^R	(Näätsaari et al. 2012)

^a These *P. pastoris* CBS7435 derived strains are marker-free knockouts^b Strains from 'Pichia Pool' of TU Graz (M. Ahmad, unpublished results)

activity, but also complicates downstream processing of the intact product as the degradation products will have similar physicochemical and affinity properties. Proteolysis may occur either during vesicular transport of recombinant protein by secretory pathway-resident proteases (Werten and de Wolf 2005; Ni et al. 2008) or in the extracellular space by proteases being secreted, cell wall-associated (Kang et al. 2000) or released into the culture medium as a result of cell disruption during high cell density cultivation (Sinha et al. 2005). Different strategies have been employed to address the proteolysis problem, namely, modifying fermentation parameters (pH, temperature and specific growth rate), changing the media composition (rich medium, addition of casamino acids or peptone as competing substrates), lowering the salt concentration and addition of soytone (Zhao et al. 2008), applying protein engineering strategies (Gustavsson et al. 2001) and engineering of the expression host to obtain protease-deficient strains (reviewed by Idiris et al. 2010 and Macauley-Patrick et al. 2005). However, in some cases, optimization of the fermentation media and protein engineering strategies failed to alleviate the proteolysis problem and tuning the expression host itself was the only viable option (Li et al. 2010). The use of protease-deficient strains such as SMD1163 ($\Delta his4 \Delta pep4 \Delta prb1$), SMD1165 ($\Delta his4 \Delta prb1$) and SMD1168 ($\Delta his4 \Delta pep4$) has been well documented for the expression of protease-sensitive proteins (Gleeson et al. 1998). *PEP4* encodes a major vacuolar aspartyl protease which is able to activate itself as well as further proteases such as carboxypeptidase Y (*PRC1*) and proteinase B (*PRB1*). The use of protease-deficient strains other than the above mentioned (e.g., *yps1*, *kex1*, *kex2*) was reported with variable success (Ni et al. 2008; Werten and de Wolf 2005; Wu et al. 2013; Yao et al. 2009). A general conclusion from these studies is that in many cases several proteases are involved in degradation events and, therefore, it is not an easy task to optimize protein expression by knocking out just a single one. However, the

pep4 and *prb1* knockout strains are still the most effective ones in preventing recombinant protein degradation, and, hence, also the most widely applied. Although it has been reported that protease-deficient strains show typically slower growth rates, lower transformation efficiencies and reduced viability (Lin-Cereghino and Lin-Cereghino 2007), experiments in our laboratory showed robust growth behaviour of 28 protease-deficient strains that were recently created (M. Ahmad, unpublished results).

Glyco-engineered strains

When yeasts such as *P. pastoris* are chosen for production of therapeutic proteins, N- and O-linked glycosylation are of tremendous relevance. Although the assembly of the core glycans, that is, (Man)₈-(GlcNAc)₂, in the ER is highly conserved in mammals and yeasts, mammals provide a much higher diversity in the ultimate glycan structure assembled in the Golgi cisternae. Yeasts, in contrast, produce high mannose glycan structures, which may lead to decreased serum half-life and may trigger allergic reactions in the human body (Ballou 1990). While in *P. pastoris* the hyper-mannosylation is not as prominent as in *S. cerevisiae*, it is still a problem that needs to be tackled, and is therefore a target for intensive strain engineering. A very detailed summary of the glycosylation machinery and the targets for glyco-engineering in different yeast species, including *P. pastoris*, has been given recently (De Pourcq et al. 2010). To sum up briefly, engineering strategies included the introduction of a *Trichoderma reesei* α -1,2-mannosidase (Callewaert et al. 2001), the knockout of the highly conserved yeast Golgi protein α -1,6-mannosyltransferase encoded by *OCH1*, which is responsible for hyperglycosylation (Choi et al. 2003; Vervecken et al. 2004), as well as co-overexpression of several glycosyltransferases and glycosidases carrying proper targeting signals (Hamilton et al. 2003). Terminally sialylated glycoproteins

produced for the first in *P. pastoris* were obtained by introducing a complex sialic acid pathway (Hamilton et al. 2006). Key to success was the correct localization of the heterologous glycosyltransferases and glycosidases in the ER and Golgi networks. Combinatorial genetic libraries and high throughput screening methods were successfully applied to find the best targeting signal/enzyme combinations for N-linked glycoengineering (Nett et al. 2011). Furthermore, a useful guide to glyco-engineering in *P. pastoris* by using the GlycoSwitch® technology was described by Jacobs et al. (2009). These strategies, altogether, enable the production of valuable biopharmaceuticals with a more homogeneous, 'humanized' N-glycosylation pattern.

However, as yeasts also carry out O-glycosylation that differs structurally from the mammalian type (Strahl-Bolsinger et al. 1999), O-glycosylation has also been an interesting target for engineering. In *P. pastoris*, O-linked glycosylation is initiated with a mannose monosaccharide, which is further elongated by α -1,2-mannose residues and finally capped with β - or phospho-mannose residues. Until lately, the engineering strategies were limited to the use of an inhibitor of the major ER located protein-O-mannosyltransferases (PMTs) as the deletion of these genes did not yield robust and viable strains. The characterization of the *P. pastoris* PMT gene family was an important step forward in O-glycosylation engineering (Nett et al. 2013). In this study, the knockout of PMTs as well as the use of PMT inhibitors led to a reduced number of O-mannosylation events and, furthermore, to reduced chain lengths of the O-glycans. A follow-up study described the production of a TNFR2:Fc¹ fusion protein carrying sialylated O-linked glycans in *P. pastoris* (Hamilton et al. 2013). Therein, an α -1,2-mannosidase as well as a protein-O-linked-mannose β -1,2-N-acetylglucosaminyl-transferase 1 (PomGnT1) were co-expressed in a *P. pastoris* strain, that was already engineered in its N-glycosylation pathway. Hence, the mannose residues were first trimmed to single O-linked mannose residues, which were then capped with N-acetylglucosamine. This structure was extended with sialic acid residues to achieve human-like O-glycan residues similar to the α -dystroglycan-type. However, there is still room for improvement, for example by engineering *P. pastoris* towards human mucin-type O-glycosylation.

Expression strategies and industrial applications

Screening for high level expression

Subsequent to the choice of suitable expression vectors and proper host strains, and transformation of the expression

cassettes, it is important to select for transformants which show high expression levels of the desired protein. Single copy transformants can be easily generated by targeting the linear expression cassettes to the *AOX1* locus resulting in gene replacement events. Ectopic integrations may simultaneously occur, however. Transformants resulting from gene replacement at the *AOX1* locus have methanol utilization slow phenotype (Mut^S) and can be easily identified by replica-plating on minimal methanol plates. The most commonly applied strategy to screen for high-yielding *P. pastoris* transformants focusses on screening for clones having multicopy integrations of the expression cassette. A recent detailed review describes the methods applied to obtain strains containing multiple expression cassettes and provides a summary of published data showing correlations between copy number and expression levels of intracellular as well as secreted proteins. It also highlights the problem of genetic instability of the integration cassettes that might be encountered when cultivating multicopy strains. Due to the highly recombinogenic nature of *P. pastoris*, expression cassettes might be excised through loop-out recombination. This effect seems to be more pronounced the more copies are integrated (Aw and Polizzi 2013).

Regarding the correlation between copy number and expression level, a number of recent studies have shown a direct correlation especially for intracellular expression (Marx et al. 2009; Vassileva et al. 2001). The direct correlation of expression level and gene copy number is, however, not necessarily valid when the protein is directed to the secretory pathway. The most commonly employed method of generating multicopy expression strains in *P. pastoris* is based on plating the transformation mixture directly on selection plates containing increasing concentrations of antibiotics (e.g., 100 to 2,000 μ g/ml of ZeocinTM). The majority of transformants will have a single copy of the expression vector integrated into the genome, and numerous clones will have to be screened to find high-copy transformants (Lin-Cereghino and Lin-Cereghino 2007). Therefore, several high-throughput methods have been established to screen a large number of clones based on small-scale cultivation in deep well plates (Mellitzer et al. 2012; Weinhandl et al. 2012; Weis et al. 2004). The selected clones, however, might not perform as well in fermenter cultivations due to different cultivation conditions. A further pronounced problem of resistance marker based screening is a high prevalence of false-positive colonies. This so-called high transformation background is supposedly caused by cell stress and cell rupture. Depending on the mechanism of antibiotic resistance conferred by the resistance marker, un-transformed cells may survive in the vicinity of ruptured transformants. This problem was addressed by constructing expression vectors based on marker gene expression driven by the weak *ARG4* promoter (*Pichia* Pool, Fig. 2). This ensures basal levels of expression, thereby allowing handlers to select single copy to

¹ Ectodomain of tumor necrosis factor 2 with crystallizable fragment of IgG1 (Fc)

multicopy strains by plating the transformants directly on low concentrations of Zeocin™ (i.e., 25 µg/ml for single copy and up to 400 µg/ml for multi-copy transformants). Thus, transformants having 1 to 20 (± 5) copies can be selected. To reduce the chances of having single copy transformants, regeneration time should be kept short and transformants should be plated directly on increased concentrations of antibiotic. By employing this method, only few transformants survive on high concentrations of antibiotic, but will most likely contain multiple copies, which can be determined by quantitative (qPCR) or Southern blot analysis (M. Ahmad, unpublished results). Performance can then be tested directly under production conditions in bioreactor cultivations instead of small-scale cultivations in deep well plates or shake flasks.

Membrane protein expression

P. pastoris has been shown to produce 15+ g of soluble recombinant protein per litre of culture intracellularly (Hasslacher et al. 1997) or in secretory mode (Werten et al. 1999). Key to such high titres is the ability of *P. pastoris* to grow to very high cell densities reaching up to 150 g cell dry weight per litre of fermentation broth in fed-batch bioreactor cultivations (Jahic et al. 2006). At very high cell densities, even proteins that are present in limited entities per single cell can be produced with reasonable volumetric yields in *P. pastoris*. Typical examples of non-abundant proteins with high scientific and commercial relevance are integral membrane proteins. Being the targets of >50 % of drugs applied on humans (Arinaminpathy et al. 2009), only very few membrane proteins have been characterized on the molecular level regarding structure–function relationships. The simple reason is that it is difficult to obtain sufficient purified membrane protein for structural and biochemical studies, unless affinity-tagged membrane proteins are obtained at reasonable yield. Actually, *P. pastoris* has been applied routinely to produce affinity-tagged membrane proteins for protein purification and subsequent biochemical studies (Cohen et al. 2005; Haviv et al. 2007; Lifshitz et al. 2007). Furthermore, *P. pastoris* has been the expression host of choice for elucidating the crystal structures of membrane proteins from diverse origins, even from higher eukaryotes (Brohawn et al. 2012; Hino et al. 2012; Ho et al. 2009).

Evolutionary proximity of a heterologous expression host and the origin of an expressed membrane protein are beneficial for successful recombinant expression (Grisshammer and Tateu 2009). In addition to the intramolecular forces and bonds, ions, cofactors and interacting proteins that stabilize soluble proteins, membrane proteins are usually interacting with and are partially also stabilized by the lipids of the surrounding bilayers (Adamian et al. 2011). As *P. pastoris* and other yeast expression hosts do significantly differ in their membrane compositions from bacterial, plant or animal cells

(Wriessnegger et al. 2007, 2009; Zinser and Daum 1995), heterologous membrane proteins may face stability issues upon expression in distantly related hosts. Thus, multiple approaches have been undertaken to improve *P. pastoris* host strains and expression conditions for membrane protein production. Applying similar tools as for the optimisation of soluble protein expression — that is, manipulation of expression conditions, addition of chemical chaperones, co-expression of chaperones or of proteins activating UPR, use of protease deficient strains, etc. — has been showing some, however often target-specific success in membrane protein expression. A novel approach is the engineering of *P. pastoris* cellular membranes for improved accommodation of heterologous membrane proteins. In the first reported example, a cholesterol-producing *P. pastoris* strain was shown to stably express an enhanced level of ligand-binding human Na,K-ATPase moieties on the cell surface (Hirz et al. 2013).

Products on — or on the way to — the market

The *P. pastoris* expression system has gained importance for industrial application as highlighted by the number of patents published on heterologous expression in and cell engineering of *P. pastoris* (Bollok et al. 2009). Products obtained by heterologous expression in *P. pastoris* have already found their way to the market, as FDA approved biopharmaceuticals or industrial enzymes have shown. The www.pichia.com web page provides a list of proteins produced in *P. pastoris* with the commercial expression system licensed by Research Corporation Technologies (RCT) and their applications: Phytase (Phytex, Sheridan, IN, USA) is applied as animal feed additive to cleave plant derived phytate, thereby providing a source of phosphate. Trypsin (Roche Applied Science, Germany) is used, for example, as protease in proteomics research to obtain peptide patterns for MS analysis. Further examples listed are nitrate reductase (The Nitrate Elimination Co., Lake Linden, MI, USA), used for water testing and treatment, phospholipase C (Verenium, San Diego, CA, USA/DSM, The Netherlands), used for degumming of vegetable oils, and Collagen (Fibrogen, San Francisco, CA, USA), used in medical research and as dermal filler. Thermo Scientific (Waltham, MA, USA) sells recombinant *Tritirachium album* Proteinase K produced in *P. pastoris*. Concerning biopharmaceuticals, a famous example is Kalbitor® (ecallantide), produced in *P. pastoris* by Dyax (Cambridge, MA, USA). Kalbitor® is a plasma kallikrein inhibitor indicated against hereditary angioedema. This product was the first biopharmaceutical to be approved by the FDA for market release in 2009 (Walsh 2010). As can be found on the web page of RCT (www.rctech.com), *Pichia*-manufactured Jetrea®, a drug used for treatment of symptomatic vitreomacular adhesion, was recently approved by the FDA and the European Commission. Other *Pichia*-

derived products provided by the Indian company Biocon are recombinant human insulin and analogues thereof (Insulin, Glargine). Products under development, such as Elastase inhibitor against Cystic fibrosis or Nanobody® ALX antibody fragments developed by Ablynx (Belgium), are also listed by Gemgross (2004) and on www.pichia.com. In 2008, Novozymes (Denmark), which found a highly active antimicrobial agent, the plectasin peptide derivative NZ2114 (Andes et al. 2009; Mygind et al. 2005), granted Sanofi-Aventis (France) an exclusive licence for the production and commercialisation of this compound in *P. pastoris*. This might be the first antimicrobial peptide approved for the market in the future.

Although not yet approved for medical use, many products can be found on the market for research purposes. GenScript (Piscataway, NJ, USA) provides recombinant cytokines and growth factors, such as human HSA-IFN- α 2b, human Stem Cell Factor SCF, murine TNF- α and ovine IFN- τ , to name just a few examples. Recombinant human angiostatin can be found for instance in the reagents offered by Sigma-Aldrich (St. Louis, MO, USA).

Future perspectives — outlook

Successful expression of many industrial enzymes as well as pharmaceutically relevant proteins has rendered the methylotrophic yeast *P. pastoris* one of the most suitable and powerful protein production host systems. It is also an emerging host for the expression of membrane proteins (Hirz et al. 2013) and of small bioactive and antimicrobial peptides, which could be a forthcoming alternative to chemical synthesis (Zhang et al. 2014). Although many basic elements of this expression system are now well developed and one can make use of a broad variety of vectors and host strains, there is still space for further optimization of protein expression and secretion, which, in many cases, will be highly dependent on the desired product. One general interest is to find effective alternatives for induction to replace methanol for industrial scale fermentations (Delic et al. 2013; Prielhofer et al. 2013; Stadlmayr et al. 2010).

Improving protein secretion performance is one of the first and foremost goals for engineering *P. pastoris*. There is still potential to increase yields, for example, by employing different secretion signals (Vadhana et al. 2013) or mutating *S. cerevisiae* α -MF (Lin-Cereghino et al. 2013). In contrast to the well-studied secretory pathway of *S. cerevisiae*, *P. pastoris* still is a black box regarding factors influencing secretion efficiency. Current studies try to identify these factors by mutagenesis approaches and screening for enhanced secretion of reporter proteins (Larsen et al. 2013; C. Winkler and H. Pichler, unpublished results). The well-developed tools for strain engineering, including marker-free integration and

deletion of desired genes, will provide a powerful set of engineered designer host strains in the near future. These will provide optimized cell factories by fine-tuned co-expression of important homologous or heterologous protein functions needed for efficient and accurate functional expression, secretion and post-translational modification of proteins. Moreover, knockout or knockdown of undesired functions such as proteolytic decay will increase product quality and process performance. Considering the scope of this review on heterologous protein expression, it was not feasible to address all possible applications for *P. pastoris* as production organism, such as metabolic engineering for production of small molecules and metabolites, or for whole-cell biocatalysis. However, developments in these fields may also be relevant for constructing improved host strains dedicated for protein production. There are several recent reviews and research articles describing advances in these fields in detail (Abad et al. 2010; Araya-Garay et al. 2012; Wriessnegger and Pichler 2013).

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Abad S, Nahalka J, Bergler G, Arnold SA, Speight R, Fotheringham I, Nidetzky B, Glieder A (2010) Stepwise engineering of a *Pichia pastoris* D-amino acid oxidase whole cell catalyst. *Microb Cell Factories* 9:24. doi:10.1186/1475-2859-9-24
- Adamian L, Naveed H, Liang J (2011) Lipid-binding surfaces of membrane proteins: evidence from evolutionary and structural analysis. *Biochim Biophys Acta* 1808:1092–1102. doi:10.1016/j.bbame.2010.12.008
- Ahn J, Hong J, Lee H, Park M, Lee E, Kim C, Choi E, Jung J, Lee H (2007) Translation elongation factor 1- α gene from *Pichia pastoris*: molecular cloning, sequence, and use of its promoter. *Appl Microbiol Biotechnol* 74:601–608. doi:10.1007/s00253-006-0698-6
- Ahn J, Hong J, Park M, Lee H, Lee E, Kim C, Lee J, Choi E, Jung J, Lee H (2009) Phosphate-responsive promoter of a *Pichia pastoris* sodium phosphate symporter. *Appl Environ Microbiol* 75:3528–3534. doi:10.1128/AEM.02913-08
- Andes D, Craig W, Nielsen LA, Kristensen HH, (2009) *In vivo* pharmacodynamic characterization of a novel plectasin antibiotic, NZ2114, in a murine infection model. *Antimicrob Agents Chemother* 53: 3003–3009. doi:10.1128/AAC.01584-08
- Araya-Garay JM, Feijoo-Siota L, Rosa-dos-Santos F, Veiga-Crespo P, Villa TG (2012) Construction of new *Pichia pastoris* X-33 strains for production of lycopene and β -carotene. *Appl Microbiol Biotechnol* 93:2483–2492. doi:10.1007/s00253-011-3764-7
- Arinaminpathy Y, Khurana E, Engelman DM, Gerstein MB (2009) Computational analysis of membrane proteins: the largest class of drug targets. *Drug Discov Today* 14:1130–1135. doi:10.1016/j.drudis.2009.08.006
- Austin RJ, Kuestner RE, Chang DK, Madden KR, Martin DB (2011) SILAC compatible strain of *Pichia pastoris* for expression of

- isotopically labeled protein standards and quantitative proteomics. *J Proteome Res* 10:5251–5259. doi:10.1021/pr200551e
- Aw R, Polizzi KM (2013) Can too many copies spoil the broth? *Microb Cell Factories* 12:128. doi:10.1186/1475-2859-12-128
- Ballou CE (1990) Isolation, characterization, and properties of *Saccharomyces cerevisiae* *mn* mutants with nonconditional protein glycosylation defects. *Methods Enzymol* 185:440–470
- Boehm T, Pirie-Shepherd S, Trinh LB, Shiloach J, Folkman J (1999) Disruption of the *KEX1* gene in *Pichia pastoris* allows expression of full-length murine and human endostatin. *Yeast* 15:563–572. doi:10.1002/(SICI)1097-0061(199905)15:7<563::AID-YEA398>3.0.CO;2-R
- Bollok M, Resina D, Valero F, Ferrer P (2009) Recent patents on the *Pichia pastoris* expression system: expanding the toolbox for recombinant protein production. *Recent Pat Biotechnol* 3:192–201. doi:10.2174/187220809789389126
- Brake AJ, Merryweather JP, Coit DG, Heberlein UA, Masiarz FR, Mullenbach GT, Urdea MS, Valenzuela P, Barr PJ (1984) Alpha-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 81:4642–4646
- Brohawn SG, del Marmol J, MacKinnon R (2012) Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K⁺ ion channel. *Science* 335:436–441. doi:10.1126/science.1213808
- Budisa N, Wenger W, Wilschi B (2010) Residue-specific global fluorination of *Candida antarctica* lipase B in *Pichia pastoris*. *Mol Biosyst* 6:1630–1639. doi:10.1039/c002256j
- Callewaert N, Laroy W, Cadirgi H, Geysens S, Saelens X, Min Jou W, Contreras R (2001) Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2- α -D-mannosidase for N-glycan engineering in *Pichia pastoris*. *FEBS Lett* 503:173–178
- Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev* 24:45–66. doi:10.1111/j.1574-6976.2000.tb00532.x
- Choi B-K, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, Miele RG, Nett JH, Wildt S, Gerngross TU (2003) Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. *Proc Natl Acad Sci U S A* 100:5022–5027. doi:10.1073/pnas.0931263100
- Ciofalo V, Barton N, Kreps J, Coats I, Shanahan D (2006) Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. *Regul Toxicol Pharmacol* 45:1–8. doi:10.1016/j.yrtph.2006.02.001
- Cohen E, Goldshleger R, Shainskaya A, Tal DM, Ebel C, le Maire M, Karlish SJD (2005) Purification of Na⁺, K⁺-ATPase expressed in *Pichia pastoris* reveals an essential role of phospholipid-protein interactions. *J Biol Chem* 280:16610–16618. doi:10.1074/jbc.M414290200
- Cregg J, Tolstorukov I (2012) *P. pastoris* ADH promoter and use thereof to direct expression of proteins. United States patent US 8,222,386
- Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985) *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* 5:3376–3385. doi:10.1128/MCB.5.12.3376
- Cregg JM, Madden KR, Barringer KJ, Thill GP, Stillman CA (1989) Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Mol Cell Biol* 9:1316–1323
- Cregg JM, Shen S, Johnson M, Waterham HR (1998) Classical genetic manipulation. *Methods Mol Biol* 103:17–26. doi:10.1385/0-89603-421-6:17
- Daly R, Hearn MTW (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *J Mol Recognit* 18:119–138. doi:10.1002/jmr.687
- Damasceno LM, Huang C-J, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. *Appl Microbiol Biotechnol* 93:31–39. doi:10.1007/s00253-011-3654-z
- de Almeida JRM, de Moraes LMP, Torres FAG (2005) Molecular characterization of the 3-phosphoglycerate kinase gene (*PGK1*) from the methylotrophic yeast *Pichia pastoris*. *Yeast* 22:725–737. doi:10.1002/yea.1243
- De Pourcq K, De Schutter K, Callewaert N (2010) Engineering of glycosylation in yeast and other fungi: current state and perspectives. *Appl Microbiol Biotechnol* 87:1617–1631. doi:10.1007/s00253-010-2721-1
- De Schutter K, Lin Y-C, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouzé P, Van de Peer Y, Callewaert N (2009) Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nat Biotechnol* 27:561–566. doi:10.1038/nbt.1544
- Delic M, Mattanovich D, Gasser B (2013) Repressible promoters — a novel tool to generate conditional mutants in *Pichia pastoris*. *Microb Cell Factories* 12:6. doi:10.1186/1475-2859-12-6
- Du M, Battles MB, Nett JH (2012) A color-based stable multi-copy integrant selection system for *Pichia pastoris* using the attenuated *ADE1* and *ADE2* genes as auxotrophic markers. *Bioeng Bugs* 3:32–37. doi:10.4161/bbug.3.1.17936
- Eiden-Plach A, Zagorc T, Heintzel T, Carius Y, Breinig F, Schmitt MJ (2004) Viral preprotoxin signal sequence allows efficient secretion of green fluorescent protein by *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. *Appl Environ Microbiol* 70:961–966. doi:10.1128/AEM.70.2.961-966.2004
- Ellis SB, Brust PF, Koutz PJ, Waters AF, Harpold MM, Gingeras TR (1985) Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Mol Cell Biol* 5:1111–1121
- Gasser B, Prielhofer R, Marx H, Maurer M, Nocon J, Steiger M, Puxbaum V, Sauer M, Mattanovich D (2013) *Pichia pastoris*: protein production host and model organism for biomedical research. *Future Microbiol* 8:191–208. doi:10.2217/fmb.12.133
- Gerngross TU (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat Biotechnol* 22:1409–1414. doi:10.1038/nbt1028
- Gleeson MA, White CE, Meininger DP, Komives EA (1998) Generation of protease-deficient strains and their use in heterologous protein expression. *Methods Mol Biol* 103:81–94. doi:10.1385/0-89603-421-6:81
- Grishammer R, Tateu CG (2009) Overexpression of integral membrane proteins for structural studies. *Q Rev Biophys* 28:315. doi:10.1017/S0033583500003504
- Guerfal M, Ryckaert S, Jacobs PP, Ameloot P, Van Craenenbroeck K, Derycke R, Callewaert N (2010) The *HAC1* gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. *Microb Cell Factories* 9:49. doi:10.1186/1475-2859-9-49
- Gustavsson M, Lehtio J, Denman S, Teeri TT, Hult K, Martinelle M (2001) Stable linker peptides for a cellulose-binding domain–lipase fusion protein expressed in *Pichia pastoris*. *Protein Eng Des Sel* 14:711–715. doi:10.1093/protein/14.9.711
- Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H, Wildt S, Gerngross TU (2003) Production of complex human glycoproteins in yeast. *Science* 301:1244–1246. doi:10.1126/science.1088166
- Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi B, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* 313:1441–1443. doi:10.1126/science.1130256
- Hamilton SR, Cook WJ, Gomathinayagam S, Burnina I, Bukowski J, Hopkins D, Schwartz S, Du M, Sharkey NJ, Bobrowicz P, Wildt S, Li H, Stadheim TA, Nett JH (2013) Production of sialylated O-linked glycans in *Pichia pastoris*. *Glycobiology* 23:1192–1203. doi:10.1093/glycob/cwt056

- Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A (2008) Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. *Nucleic Acids Res* 36:e76. doi:10.1093/nar/gkn369
- Hasslacher M, Schall M, Hayn M, Bona R, Rumbold K, Lückl J, Griengl H, Kohlwein SD, Schwab H (1997) High-level intracellular expression of hydroxynitrile lyase from the tropical rubber tree *Hevea brasiliensis* in microbial hosts. *Protein Expr Purif* 11:61–71. doi:10.1006/prep.1997.0765
- Haviv H, Cohen E, Lifshitz Y, Tal DM, Goldshleger R, Karlsh SJD (2007) Stabilization of Na(+), K(+)-ATPase purified from *Pichia pastoris* membranes by specific interactions with lipids. *Biochemistry* 46:12855–12867. doi:10.1021/bi701248y
- Hino T, Arakawa T, Iwanari H, Yurugi-Kobayashi T, Ikeda-Suno C, Nakada-Nakura Y, Kusano-Arai O, Weyand S, Shimamura T, Nomura N, Cameron AD, Kobayashi T, Hamakubo T, Iwata S, Murata T (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* 482:237–240. doi:10.1038/nature10750
- Hirz M, Richter G, Leitner E, Wriessnegger T, Pichler H (2013) A novel cholesterol-producing *Pichia pastoris* strain is an ideal host for functional expression of human Na, K-ATPase $\alpha\beta 1$ isoform. *Appl Microbiol Biotechnol* 97:9465–9478. doi:10.1007/s00253-013-5156-7
- Ho JD, Yeh R, Sandstrom A, Chorny I, Harries WEC, Robbins RA, Miercke LJW, Stroud RM (2009) Crystal structure of human aquaporin 4 at 1.8 Å and its mechanism of conductance. *Proc Natl Acad Sci U S A* 106:7437–7442. doi:10.1073/pnas.0902725106
- Idiris A, Tohda H, Kumagai H, Takegawa K (2010) Engineering of protein secretion in yeast: strategies and impact on protein production. *Appl Microbiol Biotechnol* 86:403–417. doi:10.1007/s00253-010-2447-0
- Inan M, Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. *J Biosci Bioeng* 92: 585–589. doi:10.1016/S1389-1723(01)80321-2
- Jacobs PP, Geysens S, Verweken W, Contreras R, Callewaert N (2009) Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nat Protoc* 4:58–70. doi:10.1038/nprot.2008.213
- Jahic M, Veide A, Charoenrat T, Teeri T, Enfors S-O (2006) Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. *Biotechnol Prog* 22:1465–1473. doi:10.1021/bp060171t
- Jin F, Xu X, Zhang W, Gu D (2006) Expression and characterization of a housefly cecropin gene in the methylotrophic yeast, *Pichia pastoris*. *Protein Expr Purif* 49:39–46. doi:10.1016/j.pep.2006.03.008
- Julius D, Brake A, Blair L, Kunisawa R, Thorne J (1984) Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro- α -factor. *Cell* 37: 1075–1089
- Kang HA, Choi ES, Hong WK, Kim JY, Ko SM, Sohn JH, Rhee SK (2000) Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 53:575–582
- Kato S, Ishibashi M, Tatsuda D, Tokunaga H, Tokunaga M (2001) Efficient expression, purification and characterization of mouse salivary α -amylase secreted from methylotrophic yeast, *Pichia pastoris*. *Yeast* 18:643–655. doi:10.1002/yea.714
- Khasa YP, Conrad S, Sengul M, Plautz S, Meagher MM, Inan M (2011) Isolation of *Pichia pastoris* PIR genes and their utilization for cell surface display and recombinant protein secretion. *Yeast* 28:213–226. doi:10.1002/yea.1832
- Kjeldsen T, Hach M, Balschmidt P, Havelund S, Pettersson AF, Markussen J (1998) Prepro-leaders lacking N-linked glycosylation for secretory expression in the yeast *Saccharomyces cerevisiae*. *Protein Expr Purif* 14:309–316. doi:10.1006/prep.1998.0977
- Kottmeier K, Ostermann K, Bley T, Rödel G (2011) Hydrophobin signal sequence mediates efficient secretion of recombinant proteins in *Pichia pastoris*. *Appl Microbiol Biotechnol* 91:133–141. doi:10.1007/s00253-011-3246-y
- Kranthi BV, Balasubramanian N, Rangarajan PN (2006) Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, *Pichia pastoris* and its identification as zeta crystallin. *Nucleic Acids Res* 34:4060–4068. doi:10.1093/nar/gkl577
- Kranthi BV, Kumar R, Kumar NV, Rao DN, Rangarajan PN (2009) Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in *Pichia pastoris*. *Biochim Biophys Acta* 1789:460–468. doi:10.1016/j.bbaggm.2009.05.004
- Kübler A, Schneider J, Thallinger GG, Anderl I, Wibberg D, Hajek T, Jaenicke S, Brinkrolf K, Goesmann A, Szczepanowski R, Pühler A, Schwab H, Glieder A, Pichler H (2011) High-quality genome sequence of *Pichia pastoris* CBS7435. *J Biotechnol* 154:312–320. doi:10.1016/j.jbiotec.2011.04.014
- Kumar NV, Rangarajan PN (2012) The zinc finger proteins Mxr1p and repressor of phosphoenolpyruvate carboxykinase (ROP) have the same DNA binding specificity but regulate methanol metabolism antagonistically in *Pichia pastoris*. *J Biol Chem* 287:34465–34473. doi:10.1074/jbc.M112.365304
- Kurtzman C (2009) Biotechnological strains of *Komagataella* (*Pichia*) *pastoris* are *Komagataella phaffii* as determined from multigene sequence analysis. *J Ind Microbiol Biotechnol* 36:1435–1438. doi:10.1007/s10295-009-0638-4
- Larsen S, Weaver J, de Sa CK, Bulahan R, Nguyen J, Grove H, Huang A, Low L, Tran N, Gomez S, Yau J, Ilustrisimo T, Kawilarang J, Lau J, Tranphung M, Chen I, Tran C, Fox M, Lin-Cereghino J, Lin-Cereghino GP (2013) Mutant strains of *Pichia pastoris* with enhanced secretion of recombinant proteins. *Biotechnol Lett*. doi:10.1007/s10529-013-1290-7
- Lee CC, Williams TG, Wong DWS, Robertson GH (2005) An episomal expression vector for screening mutant gene libraries in *Pichia pastoris*. *Plasmid* 54:80–85. doi:10.1016/j.plasmid.2004.12.001
- Li Z, Leung W, Yon A, Nguyen J, Perez VC, Vu J, Giang W, Luong LT, Phan T, Salazar KA, Gomez SR, Au C, Xiang F, Thomas DW, Franz AH, Lin-Cereghino J, Lin-Cereghino GP (2010) Secretion and proteolysis of heterologous proteins fused to the *Escherichia coli* maltose binding protein in *Pichia pastoris*. *Protein Expr Purif* 72: 113–124. doi:10.1016/j.pep.2010.03.004
- Liang S, Li C, Ye Y, Lin Y (2013a) Endogenous signal peptides efficiently mediate the secretion of recombinant proteins in *Pichia pastoris*. *Biotechnol Lett* 35:97–105. doi:10.1007/s10529-012-1055-8
- Liang S, Zou C, Lin Y, Zhang X, Ye Y (2013b) Identification and characterization of P GCW14 : a novel, strong constitutive promoter of *Pichia pastoris*. *Biotechnol Lett* 35:1865–1871. doi:10.1007/s10529-013-1265-8
- Lifshitz Y, Petrovich E, Haviv H, Goldshleger R, Tal DM, Garty H, Karlsh SJD (2007) Purification of the human $\alpha 2$ Isoform of Na, K-ATPase expressed in *Pichia pastoris*. Stabilization by lipids and FXYD1. *Biochemistry* 46:14937–14950. doi:10.1021/bi701812c
- Lin-Cereghino J, Lin-Cereghino GP (2007) Vectors and strains for expression. *Methods Mol Biol* 389:11–26. doi:10.1007/978-1-59745-456-8_2
- Lin-Cereghino GP, Lin-Cereghino J, Jay Sunga A, Johnson MA, Lim M, Gleeson MAG, Cregg JM (2001) New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*. *Gene* 263:159–169. doi:10.1016/S0378-1119(00)00576-X
- Lin-Cereghino GP, Godfrey L, de la Cruz BJ, De JS, Khuongsathiene S, Tolstorukov I, Yan M, Lin-Cereghino J, Veenhuis M, Subramani S, Cregg JM (2006) Mxr1p, a key regulator of the methanol utilization

- pathway and peroxisomal genes in *Pichia pastoris*. Mol Cell Biol 26:883–897. doi:10.1128/MCB.26.3.883-897.2006
- Lin-Cereghino GP, Stark CM, Kim D, Chang JWJ, Shaheen N, Poerwanto H, Agari K, Moua P, Low LK, Tran N, Huang AD, Nattestad M, Oshiro KT, Chavan A, Tsai JW, Lin-Cereghino J (2013) The effect of α -mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. Gene 519:311–317. doi:10.1016/j.gene.2013.01.062
- Lünsdorf H, Gurramkonda C, Adnan A, Khanna N, Rinas U (2011) Virus-like particle production with yeast: ultrastructural and immunocytochemical insights into *Pichia pastoris* producing high levels of the hepatitis B surface antigen. Microb Cell Factories 10:48. doi:10.1186/1475-2859-10-48
- Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–270. doi:10.1002/yea.1208
- Marx H, Mecklenbräuker A, Gasser B, Sauer M, Mattanovich D (2009) Directed gene copy number amplification in *Pichia pastoris* by vector integration into the ribosomal DNA locus. FEMS Yeast Res 9:1260–1270. doi:10.1111/j.1567-1364.2009.00561.x
- Mattanovich D, Callewaert N, Rouzé P, Lin Y-C, Graf A, Redl A, Tiels P, Gasser B, De Schutter K (2009a) Open access to sequence: browsing the *Pichia pastoris* genome. Microb Cell Factories 8:53. doi:10.1186/1475-2859-8-53
- Mattanovich D, Graf A, Stadlmann J, Dragosits M, Redl A, Maurer M, Kleinheinz M, Sauer M, Altmann F, Gasser B (2009b) Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. Microb Cell Factories 8:29. doi:10.1186/1475-2859-8-29
- Mellitzer A, Glieder A, Weis R, Reisinger C, Flicker K (2012) Sensitive high-throughput screening for the detection of reducing sugars. Biotechnol J 7:155–162. doi:10.1002/biot.201100001
- Menendez J, Valdes I, Cabrera N (2003) The *ICL1* gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. Yeast 20:1097–1108. doi:10.1002/yea.1028
- Moir DT, Dumais DR (1987) Glycosylation and secretion of human alpha-1-antitrypsin by yeast. Gene 56:209–217
- Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sönksen CP, Ludvigsen S, Raventos D, Buskov S, Christensen B, De Maria L, Taboureau O, Yaver D, Elvig-Jørgensen SG, Sørensen MV, Christensen BE, Kjaerulff S, Frimodt-Møller N, Lehrer RI, Zasloff M, Kristensen H-H (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 437:975–980. doi:10.1038/nature04051
- Näätsaari L, Mistlberger B, Ruth C, Hajek T, Hartner FS, Glieder A (2012) Deletion of the *Pichia pastoris* *KU70* homologue facilitates platform strain generation for gene expression and synthetic biology. PLoS ONE 7:e39720. doi:10.1371/journal.pone.0039720
- Nett J (2010) Yeast strains for protein production. United States patent US 2010/0279348
- Nett JH, Germgross TU (2003) Cloning and disruption of the *PpURA5* gene and construction of a set of integration vectors for the stable genetic modification of *Pichia pastoris*. Yeast 20:1279–1290. doi:10.1002/yea.1049
- Nett JH, Hodel N, Rausch S, Wildt S (2005) Cloning and disruption of the *Pichia pastoris* *ARG1*, *ARG2*, *ARG3*, *HIS1*, *HIS2*, *HIS5*, *HIS6* genes and their use as auxotrophic markers. Yeast 22:295–304. doi:10.1002/yea.1202
- Nett JH, Stadheim TA, Li H, Bobrowicz P, Hamilton SR, Davidson RC, Choi B-K, Mitchell T, Bobrowicz B, Rittenhour A, Wildt S, Germgross TU (2011) A combinatorial genetic library approach to target heterologous glycosylation enzymes to the endoplasmic reticulum or the Golgi apparatus of *Pichia pastoris*. Yeast 28:237–252. doi:10.1002/yea.1835
- Nett JH, Cook WJ, Chen M-T, Davidson RC, Bobrowicz P, Kett W, Brevnova E, Potgieter TI, Mellon MT, Prinz B, Choi B-K, Zha D, Burnina I, Bukowski JT, Du M, Wildt S, Hamilton SR (2013) Characterization of the *Pichia pastoris* protein-O-mannosyltransferase gene family. PLoS ONE 8:e68325. doi:10.1371/journal.pone.0068325
- Ni Z, Zhou X, Sun X, Wang Y, Zhang Y (2008) Decrease of hirudin degradation by deleting the *KEX1* gene in recombinant *Pichia pastoris*. Yeast 25:1–8. doi:10.1002/yea.1542
- Ohi H, Miura M, Hiramatsu R, Ohmura T (1994) The positive and negative *cis*-acting elements for methanol regulation in the *Pichia pastoris* *AOX2* gene. Mol Gen Genet 243:489–499
- Oka C, Tanaka M, Muraki M, Harata K, Suzuki K, Jigami Y (1999) Human lysozyme secretion increased by alpha-factor pro-sequence in *Pichia pastoris*. Biosci Biotechnol Biochem 63:1977–1983. doi:10.1271/bbb.63.1977
- Paifer E, Margolles E, Cremata J, Montesino R, Herrera L, Delgado JM (1994) Efficient expression and secretion of recombinant alpha amylase in *Pichia pastoris* using two different signal sequences. Yeast 10:1415–1419. doi:10.1002/yea.320101104
- Parua PK, Ryan PM, Trang K, Young ET (2012) *Pichia pastoris* 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction. Mol Microbiol 85:282–298. doi:10.1111/j.1365-2958.2012.08112.x
- Payne WE, Gannon PM, Kaiser CA (1995) An inducible acid phosphatase from the yeast *Pichia pastoris*: characterization of the gene and its product. Gene 163:19–26. doi:10.1016/0378-1119(95)00379-K
- Polupanov AS, Nazarko VY, Sibimiy AA (2012) *Gss1* protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, pexophagy and catabolite repression. Int J Biochem Cell Biol 44:1906–1918. doi:10.1016/j.biocel.2012.07.017
- Prielhofer R, Maurer M, Klein J, Wenger J, Kiziak C, Gasser B, Mattanovich D (2013) Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. Microb Cell Factories 12:5. doi:10.1186/1475-2859-12-5
- Qin X, Qian J, Yao G, Zhuang Y, Zhang S, Chu J (2011) GAP promoter library for fine-tuning of gene expression in *Pichia pastoris*. Appl Environ Microbiol 77:3600–3608. doi:10.1128/AEM.02843-10
- Raemaekers RJM, de Muro L, Gatehouse JA, Fordham-Skelton AP (1999) Functional phytohemagglutinin (PHA) and *Galanthus nivalis* agglutinin (GNA) expressed in *Pichia pastoris*. Correct N-terminal processing and secretion of heterologous proteins expressed using the PHA-E signal peptide. Eur J Biochem 265:394–403. doi:10.1046/j.1432-1327.1999.00749.x
- Rakestraw JA, Sazinsky SL, Piatetsi A, Antipov E, Wittrup KD (2009) Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in *Saccharomyces cerevisiae*. Biotechnol Bioeng 103:1192–1201. doi:10.1002/bit.22338
- Sha C, Yu X-W, Li F, Xu Y (2013) Impact of gene dosage on the production of lipase from *Rhizopus chinensis* CCTCC M201021 in *Pichia pastoris*. Appl Biochem Biotechnol 169:1160–1172. doi:10.1007/s12010-012-0050-9
- Shen S, Sulter G, Jeffries TW, Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. Gene 216:93–102
- Shi X-L, Feng M-Q, Shi J, Shi Z-H, Zhong J, Zhou P (2007) High-level expression and purification of recombinant human catalase in *Pichia pastoris*. Protein Expr Purif 54:24–29. doi:10.1016/j.pep.2007.02.008
- Sigoillot M, Brockhoff A, Lescop E, Poirier N, Meyerhof W, Briand L (2012) Optimization of the production of gumarin, a sweet-taste-suppressing protein, secreted by the methylotrophic yeast *Pichia pastoris*. Appl Microbiol Biotechnol 96:1253–1263. doi:10.1007/s00253-012-3897-3
- Sinha J, Plantz BA, Inan M, Meagher MM (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant

- ovine interferon-tau. *Biotechnol Bioeng* 89:102–112. doi:10.1002/bit.20318
- Skipper N, Sutherland M, Davies RW, Kilburn D, Miller RC, Warren A, Wong R (1985) Secretion of a bacterial cellulase by yeast. *Science* 230:958–960. doi:10.1126/science.230.4728.958
- Stadlmayr G, Mecklenbräuker A, Rothmüller M, Maurer M, Sauer M, Mattanovich D, Gasser B (2010) Identification and characterisation of novel *Pichia pastoris* promoters for heterologous protein production. *J Biotechnol* 150:519–529. doi:10.1016/j.jbiotec.2010.09.957
- Staley CA, Huang A, Nattestad M, Oshiro KT, Ray LE, Mulye T, Li ZH, Le T, Stephens JJ, Gomez SR, Moy AD, Nguyen JC, Franz AH, Lin-Cereghino J, Lin-Cereghino GP (2012) Analysis of the 5' untranslated region (5'UTR) of the alcohol oxidase 1 (*AOX1*) gene in recombinant protein expression in *Pichia pastoris*. *Gene* 496:118–127. doi:10.1016/j.gene.2012.01.006
- Strahl-Bolsinger S, Gentzsch M, Tanner W (1999) Protein O-mannosylation. *Biochim Biophys Acta Gen Subj* 1426:297–307. doi:10.1016/S0304-4165(98)00131-7
- Takagi S, Tsutsumi N, Terui Y, Kong XY (2008) Method for methanol independent induction from methanol inducible promoters in *Pichia*. United States patent US 8,143,023
- Thompson CA (2010) FDA approves kallikrein inhibitor to treat hereditary angioedema. *Am J Health Syst Pharm* 67:93. doi:10.2146/news100005
- Thor D, Xiong S, Orazem CC, Kwan A-C, Cregg JM, Lin-Cereghino J, Lin-Cereghino GP (2005) Cloning and characterization of the *Pichia pastoris* *MET2* gene as a selectable marker. *FEMS Yeast Res* 5:935–942. doi:10.1016/j.femsyr.2005.03.009
- Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR (1987a) Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res* 15:3859–3876
- Tschopp JF, Sverlow G, Kosson R, Craig W, Grinna L (1987b) High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. *Nat Biotechnol* 5:1305–1308. doi:10.1038/nbt1287-1305
- Uchima CA, Arioka M (2012) Expression and one-step purification of recombinant proteins using an alternative episomal vector for the expression of N-tagged heterologous proteins in *Pichia pastoris*. *Biosci Biotechnol Biochem* 76:368–371. doi:10.1271/bbb.110628
- Vadhana AKP, Samuel P, Berin RM, Krishna J, Kamatchi K, Meenakshisundaram S (2013) Improved secretion of *Candida antarctica* lipase B with its native signal peptide in *Pichia pastoris*. *Enzym Microb Technol* 52:177–183. doi:10.1016/j.enzmictec.2013.01.001
- van der Heide M, Hollenberg CP, van der Klei IJ, Veenhuis M (2002) Overproduction of BiP negatively affects the secretion of *Aspergillus niger* glucose oxidase by the yeast *Hansenula polymorpha*. *Appl Microbiol Biotechnol* 58:487–494. doi:10.1007/s00253-001-0907-2
- Vassileva A, Chugh DA, Swaminathan S, Khanna N (2001) Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Protein Expr Purif* 21:71–80. doi:10.1006/prep.2000.1335
- Vervecken W, Kaigorodov V, Callewaert N, Geysens S, De Vusser K, Contreras R (2004) In vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. *70:2639–46*. doi:10.1128/AEM.70.5.2639
- Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. *Nat Biotechnol* 30:385–404. doi:10.1016/j.nbt.2012.11.010
- Walsh G (2010) Biopharmaceutical benchmarks 2010. *Nat Biotechnol* 28:917–924. doi:10.1038/nbt0910-917
- Waterham HR, de Vries Y, Russel KA, Xie W, Veenhuis M, Cregg JM (1996) The *Pichia pastoris* *PER6* gene product is a peroxisomal integral membrane protein essential for peroxisome biogenesis and has sequence similarity to the Zellweger syndrome protein PAF-I. *Mol Cell Biol* 16:2527–2536
- Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 186:37–44. doi:10.1016/S0378-1119(96)00675-0
- Waters MG, Evans EA, Blobel G (1988) Prepro-alpha-factor has a cleavable signal sequence. *J Biol Chem* 263:6209–6214
- Weinhandl K, Winkler M, Glieder A, Camattari A (2012) A novel multi-enzymatic high throughput assay for transaminase activity. *Tetrahedron* 68:7586–7590. doi:10.1016/j.tet.2012.06.058
- Weiss R, Luiten R, Skranc W, Schwab H, Wubboldts M, Glieder A (2004) Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *FEMS Yeast Res* 5:179–189. doi:10.1016/j.femsyr.2004.06.016
- Weiss HM, Haase W, Michel H, Reiländer H (1995) Expression of functional mouse 5-HT_{5A} serotonin receptor in the methylotrophic yeast *Pichia pastoris*: pharmacological characterization and localization. *FEBS Lett* 377:451–456
- Werten MWT, de Wolf FA (2005) Reduced proteolysis of secreted gelatin and Yps1-mediated alpha-factor leader processing in a *Pichia pastoris* *kex2* disruptant. *Appl Environ Microbiol* 71:2310–2317. doi:10.1128/AEM.71.5.2310-2317.2005
- Werten MWT, van den Bosch TJ, Wind RD, Mooibroek H, de Wolf FA (1999) High-yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast* 15:1087–1096. doi:10.1002/(SICI)1097-0061(199908)15:11<1087::AID-YEA436>3.0.CO;2-F
- Whyteside G, Nor RM, Alcocer MJC, Archer DB (2011) Activation of the unfolded protein response in *Pichia pastoris* requires splicing of a *HAC1* mRNA intron and retention of the C-terminal tail of Hac1p. *FEBS Lett* 585:1037–1041. doi:10.1016/j.febslet.2011.02.036
- Wriessnegger T, Pichler H (2013) Yeast metabolic engineering—targeting sterol metabolism and terpenoid formation. *Prog Lipid Res* 52:277–293. doi:10.1016/j.plipres.2013.03.001
- Wriessnegger T, Gübitz G, Leitner E, Ingolic E, Cregg J, de la Cruz BJ, Daum G (2007) Lipid composition of peroxisomes from the yeast *Pichia pastoris* grown on different carbon sources. *Biochim Biophys Acta* 1771:455–461. doi:10.1016/j.bbalip.2007.01.004
- Wriessnegger T, Leitner E, Beleggratis MR, Ingolic E, Daum G (2009) Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*. *Biochim Biophys Acta* 1791:166–172. doi:10.1016/j.bbalip.2008.12.017
- Wu M, Shen Q, Yang Y, Zhang S, Qu W, Chen J, Sun H, Chen S (2013) Disruption of *YPS1* and *PEP4* genes reduces proteolytic degradation of secreted HSA/PTH in *Pichia pastoris* GS115. *J Ind Microbiol Biotechnol* 40:589–599. doi:10.1007/s10295-013-1264-8
- Xuan Y, Zhou X, Zhang W, Zhang X, Song Z, Zhang Y (2009) An upstream activation sequence controls the expression of *AOX1* gene in *Pichia pastoris*. *FEMS Yeast Res* 9:1271–1282. doi:10.1111/j.1567-1364.2009.00571.x
- Yang S, Kuang Y, Li H, Liu Y, Hui X, Li P, Jiang Z, Zhou Y, Wang Y, Xu A, Li S, Liu P, Wu D (2013) Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P1' site. *PLoS ONE* 8:e75347. doi:10.1371/journal.pone.0075347
- Yao XQ, Zhao HL, Xue C, Zhang W, Xiong XH, Wang ZW, Li XY, Liu ZM (2009) Degradation of HSA-AX15(R13K) when expressed in *Pichia pastoris* can be reduced via the disruption of *YPS1* gene in this yeast. *J Biotechnol* 139:131–136. doi:10.1016/j.jbiotec.2008.09.006
- Yoshimasu MA, Ahn J-K, Tanaka T, Yada RY (2002) Soluble expression and purification of porcine pepsinogen from *Pichia pastoris*. *Protein Expr Purif* 25:229–236
- Yu XW, Sha C, Guo YL, Xiao R, Xu Y (2013) High-level expression and characterization of a chimeric lipase from *Rhizopus oryzae* for

- biodiesel production. *Biotechnol Biofuels* 6:29. doi:10.1186/1754-6834-6-29
- Zhang Y, Teng D, Mao R, Wang X, Xi D, Hu X, Wang J (2014) High expression of a plectasin-derived peptide NZ2114 in *Pichia pastoris* and its pharmacodynamics, postantibiotic and synergy against *Staphylococcus aureus*. *Appl Microbiol Biotechnol* 98:681–694. doi:10.1007/s00253-013-4881-2
- Zhao HL, Xue C, Wang Y, Yao XQ, Liu ZM (2008) Increasing the cell viability and heterologous protein expression of *Pichia pastoris* mutant deficient in *PMR1* gene by culture condition optimization. *Appl Microbiol Biotechnol* 81:235–241. doi:10.1007/s00253-008-1666-0
- Zinser E, Daum G (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. *Yeast* 11:493–536. doi:10.1002/yea.320110602

Ex. 1030 Haon 2015



Recombinant protein production facility for fungal biomass-degrading enzymes using the yeast *Pichia pastoris*

Mireille Haon^{1,2}, Sacha Grisel^{1,2}, David Navarro^{1,2}, Antoine Gruet^{1,2,3}, Jean-Guy Berrin^{1,2*} and Christophe Bignon^{4,5*}

OPEN ACCESS

Edited by:

Vijai Kumar Gupta,
National University of Ireland Galway,
Ireland

Reviewed by:

Evangelos Topakas,
National Technical University
of Athens, Greece
Paul Christakopoulos,
Lulea University of Technology,
Sweden

*Correspondence:

Jean-Guy Berrin,
Aix-Marseille Université, Polytech
Marseille, UMR1163 Biodiversité et
Biotechnologie Fongiques, 13288
Marseille, France
jean-guy.berrin@univ-amu.fr;
Christophe Bignon,
Architecture et Fonction des
Macromolécules Biologiques,
CNRS-Aix-Marseille University
UMR 7257, Case 932 Campus
de Luminy, 163 Avenue de Luminy,
Marseille Cedex 09, France
bignon@afmb.univ-mrs.fr

Specialty section:

This article was submitted to
Microbiotechnology, Ecotoxicology
and Bioremediation,
a section of the journal
Frontiers in Microbiology

Received: 24 July 2015

Accepted: 07 September 2015

Published: 23 September 2015

Citation:

Haon M, Grisel S, Navarro D, Gruet A,
Berrin J-G and Bignon C (2015)
Recombinant protein production
facility for fungal biomass-degrading
enzymes using the yeast *Pichia*
pastoris. *Front. Microbiol.* 6:1002.
doi: 10.3389/fmicb.2015.01002

¹ INRA, UMR 1163 Biodiversité et Biotechnologie Fongiques, Marseille, France, ² Aix-Marseille Université, Polytech Marseille, UMR1163 Biodiversité et Biotechnologie Fongiques, Marseille, France, ³ Laboratory of Protein and Nucleic Acid Chemistry, The Rockefeller University, New York, NY, USA, ⁴ Architecture et Fonction des Macromolécules Biologiques, CNRS-Aix-Marseille University UMR 7257, Marseille, France, ⁵ INRA, USC 1408 AFMB, Marseille, France

Filamentous fungi are the predominant source of lignocellulolytic enzymes used in industry for the transformation of plant biomass into high-value molecules and biofuels. The rapidity with which new fungal genomic and post-genomic data are being produced is vastly outpacing functional studies. This underscores the critical need for developing platforms dedicated to the recombinant expression of enzymes lacking confident functional annotation, a prerequisite to their functional and structural study. In the last decade, the yeast *Pichia pastoris* has become increasingly popular as a host for the production of fungal biomass-degrading enzymes, and particularly carbohydrate-active enzymes (CAZymes). This study aimed at setting-up a platform to easily and quickly screen the extracellular expression of biomass-degrading enzymes in *P. pastoris*. We first used three fungal glycoside hydrolases (GHs) that we previously expressed using the protocol devised by Invitrogen to try different modifications of the original protocol. Considering the gain in time and convenience provided by the new protocol, we used it as basis to set-up the facility and produce a suite of fungal CAZymes (GHs, carbohydrate esterases and auxiliary activity enzyme families) out of which more than 70% were successfully expressed. The platform tasks range from gene cloning to automated protein purifications and activity tests, and is open to the CAZyme users' community.

Keywords: recombinant proteins, expression platform, CAZymes, *Pichia pastoris*, factorial approach, automation, glycoside hydrolase

Introduction

Lignocellulosic biomass is recognized as a sustainable source of mixed sugars for producing second generation biofuels by fermentation, and for the synthesis of biomaterials (Lynd et al., 2008). In industry, saccharification of lignocellulose is performed enzymatically using fungal enzyme cocktails (Himmel et al., 2007). Due to the recalcitrant nature of lignocellulosic biomass, the development of more efficient fungal enzyme preparations is an active research field with the sequencing of hundreds of fungal genomes at the US Department of Energy Joint Genome Institute 1,000 fungal genome project, (Grigoriev et al., 2014). This wealth

Abbreviations: AA, auxiliary activities; CAZy, carbohydrate-active enzymes; CE, carbohydrate esterase; GH, glycoside hydrolase; kDa, kiloDalton; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

of genomic information opens new horizons and opportunities to improve the enzymatic conversion of biomass.

However, due to throughput differences the production of recombinant fungal proteins lags far behind that of data generated by -omic approaches, which creates a bottleneck in the process of unraveling the function of novel CAZymes. Although historically filamentous fungi (e.g., *Trichoderma reesei*, *Aspergillus niger*) and other yeast (e.g., *Saccharomyces cerevisiae*, *Yarrowia lipolytica*) have been used for expressing native or recombinant fungal enzymes (Dashtban et al., 2009), the yeast *Pichia pastoris* has become the premier example of yeast species used for the production of recombinant proteins (Cregg et al., 2000; Damasceno et al., 2012). Advantages of this yeast include the use of efficient and tightly regulated promoters, higher expression levels for lower cost than insect and mammalian cells. Compared to prokaryotic expression, it provides better protein folding, allows post-translational modifications (N- and O-glycosylation, methylation, and acylation), and is able to secrete high levels of recombinant proteins in the culture medium. In addition, it secretes very low levels of endogenous proteins none of which has been reported to be active on lignocellulosic biomass, which simplifies protein purification.

Finally, because filamentous fungi and yeast are phylogenetically close, *P. pastoris* is the organism of choice for expressing different classes of active fungal CAZymes, such as GHs (Bauer et al., 2006; Couturier et al., 2011a,b), CE (Topakas et al., 2010, 2012) and AA enzymes [i.e., lytic polysaccharide mono-oxygenases (Kittl et al., 2012; Bey et al., 2013)]. Having these many advantages over other expression systems makes *P. pastoris* an organism of choice for the production of industrial enzymes (Rabert et al., 2013).

Despite obvious advantages, we have found that heterologous expression of fungal enzymes in *P. pastoris* remained time-consuming, and reasoned that protocols for transformation, screening of transformants, cultures, purification of recombinant proteins could benefit from streamlining to try to increase protein production-throughput. In this study, we have used in-house fungal genes encoding CAZymes [GH5, GH11, GH45 (Couturier et al., 2011a,b)] that we previously successfully expressed in *P. pastoris* using pPICZ α inducible vector, to establish a simpler standard protocol. In this paper, we have analyzed, and modified whenever possible, different steps of the protocol set-up by Invitrogen for expressing proteins in *P. pastoris* from the design of expression constructs to the assay of purified proteins, with the ultimate goal of setting-up a lab-scale recombinant protein production facility. This platform now makes the link between upstream expert annotation of CAZymes (www.cazy.org) (Lombard et al., 2014) and downstream automated assay of enzymatic activities (Lafond et al., 2012) and crystallogenesis (Sulzenbacher et al., 2002).

Materials and Methods

Enzymes

Taq (screening purpose) and Platinum pfx (cloning purpose) DNA polymerases (Invitrogen) were used in PCR experiments.

Expression Constructs

Coding sequences to express were PCR amplified, and then inserted into Invitrogen vectors pPICZ α A (Couturier et al., 2011a,b), pGAPZ α A and pBGP1 (Sasagawa et al., 2011) (this study) inframe with N-terminal yeast α -secretion factor and C-terminal His₆ tag encoding sequences. Plasmid pBGP1 was obtained by removing the Gateway cassette from pBGP1-DEST (Sasagawa et al., 2011) by XhoI/XbaI restriction digestion. The primers used were: GGGGCTAAAGAACTCGAGAAAAGAG AGGCTGAAGCTCTCCCCCAAGCACAAAGGTG and TTTTC TAGACCCGCGGGAGAGCATTGATAG for *Podospira anserine* GH5 (GenBank: HM357135.1), GGGGCTAAAGAA CTCGAGAAAAGAGAGGCTGAAGCTGCCCCCGGTGAGCT TCCT and TTTTCTAGACCGTGTGTCTGGACATAAATGTC for *P. anserine* GH11 (GenBank: HM357137.2), and GGGGCTA AAGAACTCGAGAAAAGAGAGGCTGAAGCTGATGTCCCA CTTTGGGGCCAATG and TCTAGACCTTCGTCCGTACGAG CACA for *P. pastoris* GH45 (GenBank: CAY71902.1). Except pBGP1, resulting recombinant expression plasmids were linearized with PmeI (pPICZ α A) or AvrII (pGAPZ α A) restriction enzymes.

Preparation of Frozen Electrocompetent Cells and Transformation

Cells were grown as described by Invitrogen (easyselect_man.pdf) with the following modification: when OD₆₀₀ was about 1.4, cells recovered from 250 ml culture were resuspended in 100 ml YPD supplemented with 20 ml 1 M HEPES pH8 and 2.5 ml 1 M DTT, and stored at 30°C for 15'. After washing as described in easyselect_man.pdf, they were eventually resuspended in 0.5 ml of cold 1 M sorbitol and 60 μ l aliquots were frozen at -80°C.

Electroporation was performed as described in easyselect_man.pdf using 60 μ l of thawed electrocompetent cells.

Liquid Phase Selection and Expression Screening

General Strategy

Both were performed in 24-wells deepwell plates. Indicated volumes are for one well. An aliquot (200 μ l) of sorbitol cell suspension was used to inoculate 2 ml YPD supplemented with 100 μ g/ml Zeocin, which was then incubated for 3 days at 30°C under 225 rpm rotary shaking. This step has two aims: it selects transformed cells as do Zeocin-containing agar plates, and serves as a pre-culture for the following recombinant protein expression screening. The latter was performed by inoculating 5 ml Zeocin-free growth medium with 50 μ l pre-culture and then incubating at 30°C under shaking. At the end of the expression period, culture supernatants were recovered by centrifugation for 5' at 4000 g and their protein content analyzed by SDS-PAGE and/or enzymatic activity assay.

Constitutive Expression (pGAPZ α A)

Protein expression screening was performed by inoculating 5 ml YPD with 50 μ l pre-culture and then incubating for 4 days at 30°C under shaking.

Inducible expression (pPICZ α A)

Protein expression screening was performed by inoculating 5 ml BMGY (easyselect_man.pdf) with 50 μ l pre-culture, and then incubating overnight at 30°C under shaking. The plate was then spun as above and the cell pellet was resuspended in 1 ml BMMY (easyselect_man.pdf). The culture medium was daily supplemented with 3% methanol for 4 days.

Automated Purification of Recombinant Proteins

Non-Automated Pre-Purification Steps

(i) At the end of the expression period, 24-wells deepwell plates were spun for 5' at 4000 g to pellet the cells. Supernatants were transferred well to well to another 24-wells deepwell plate, and each well was supplemented with 1/10 supernatant volume of 250 mM Tris pH8. (ii) Two hundred microliters of a 50% suspension of Ni SepharoseTM High Performance (GE Healthcare 17-5268-02) were loaded into each well of a 96-wells deepwell filter plate (AcroPrepTM Advance 1 ml, 1 μ m Glass Fiber, PALL PN 8131). (iii) 24-wells deepwell plates containing the supernatants were put on a TECAN Freedom Evo robot, and the 96-wells deepwell filter plate on the first manifold of the same robot (Navarro et al., 2010).

Automated Steps

Indicated volumes are for one well. The automate removed ethanol from Sepharose beads by vacuum aspiration for 30'' at -50 millibars (mb), and then added 200 μ l buffer A (10 mM Tris pH7.8, 150 mM NaCl and 10 mM imidazole). After 2', buffer A was eliminated by vacuum aspiration for 30'' at -50 mb. This step was repeated once. The robot transferred 800 μ l of each culture supernatant from the 24-wells deepwell plate to each well of the 96-wells deepwell filter plate. After 3' incubation, the supernatant was eliminated by vacuum aspiration for 60'' at -50 mb. If more than 1 ml supernatant was to be processed, this step was repeated the required number of times. At the end of the binding step, the robot washed the beads twice with buffer A as above, and then dried them by an additional 5'' -700 mb vacuum aspiration. The robot transferred the filter plate on top of a 96-wells microplate that had been put on the second manifold of the robot beforehand, and added 200 μ l buffer B (10 mM Tris pH7.8, 150 mM NaCl and 500 mM imidazole) to each well of the filter plate. After 5' incubation, the robot transferred the elution volume from each well of the filter plate to the corresponding well of the 96-wells microplate underneath by two successive vacuum aspirations for 20'' at -50 mb. Complete recovery of elution volume was achieved by performing a second vacuum aspiration for 5'' at -700 mb. The filter plate was then transferred back to the first manifold where beads were regenerated by performing the following operations. (i) Buffer B (200 μ l) was added and after 2' incubation removed by vacuum aspiration for 30'' at -50 mb. This step was repeated once. (ii) Buffer A (200 μ l) was added and after 2' incubation removed by vacuum aspiration for 30'' at -50 mb. (iii) Finally, beads were supplemented in 400 μ l of 20% ethanol. The filter plate was sandwiched between two adhesive aluminum sheets (Dutscher reference 106524) and stored at 4°C.

Enzymatic Activity Assays

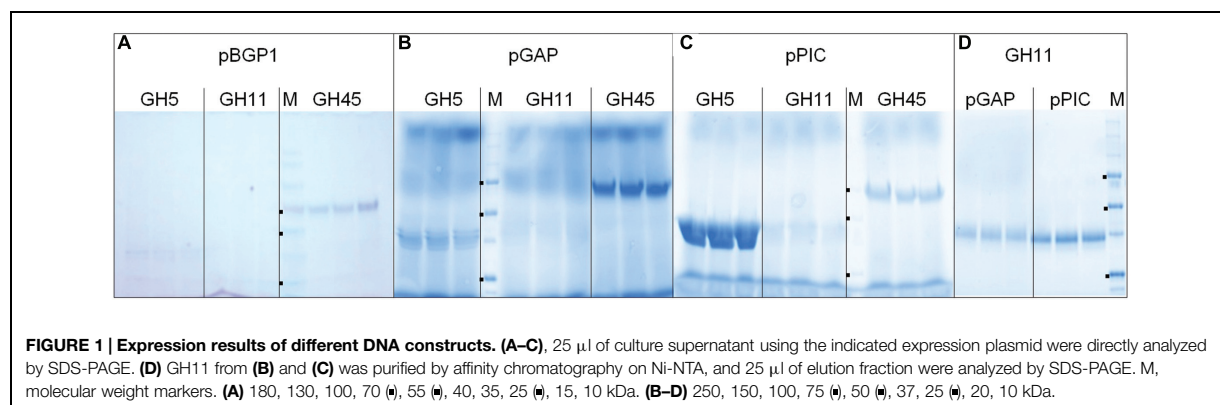
Indicated volumes are for one well. GH5, GH11, and GH45 enzymatic activities were assayed by measuring the hydrolysis of 1% azo-glucomannan, azo-xylan, and azo-CM-cellulose (Megazyme), respectively. The assay is based on GH-dependent hydrolysis of a dyed substrate that produces low-molecular weight dyed fragments which remain in solution on addition of a precipitant solution. High-molecular weight material is removed by centrifugation and the color of the supernatant is measured. In practice, culture supernatants or purified enzymes (10 μ l) were added to 50 μ l of azo-substrate solution in 50 mM sodium acetate pH4.8 in 96-well plates. The latter were heat-sealed with aluminum sheet using the robot PlateLoc sealer, and incubated at 40°C for 2 h under 800 rpm rotary shaking. Ethanol (150 μ l) was added to precipitate non-hydrolysed substrate. After 10' at room temperature, plates were centrifuged at 4000 g for 5'. Supernatants were collected and their optical density measured at 590 nm. Activities were expressed as percentage of azo-substrate used. The 100% value was the OD₅₉₀ of a well to which no ethanol and no enzyme had been added.

Results and Discussion

In a previous work, three GH (GH5, GH11, and GH45) were successfully expressed in *P. pastoris* by strictly following the protocol proposed by Invitrogen (easyselect_man.pdf). Analysis of the original protocol resulted in a list of different steps that could be subject of optimization in terms of time, cost, or convenience. The list was made of: (1) using frozen competent cells instead of extemporaneously preparing competent cells; (2) using less plasmid for electroporation; (3) skipping the selection of recombinant clones on plate; (4) replacing tubes and flasks with deep-well plates in the screening process. In addition to simplifying the original protocol, the purification of secreted His-tagged proteins was automated to increase throughput.

Expression Constructs

In a first trial, GH5, GH11, and GH45 coding sequences were sub-cloned by restriction/ligation (R/L) into episomal pBGP1 (Sasagawa et al., 2011) and integrative pGAPZ α A and pPICZ α A expression vectors as described in Section "Materials and Methods," and recombinant expression was evaluated by SDS-PAGE in the supernatant of culture media. Using integrative vectors was expected to result in genetically stable *P. pastoris* strains as they are integrated into the yeast chromosome at the cost of more laborious screening, whereas an episomal vector could be isolated by a simple plasmid preparation procedure. In addition, episomal vectors have higher transformation efficiencies and more than one copy per cell. It was therefore of interest to assess whether these features were correlated to recombinant protein expression level. Results are reported in **Figures 1A–C**. Expression of GH5 and GH45 was plasmid-dependant: GH5 was better expressed by pPICZ α A than by pGAPZ α A whereas it was the opposite for GH45. No GH11 was visible in the culture medium of any plasmid condition. To check whether GH11 was expressed, it was purified by affinity



chromatography on Ni-NTA as described in Section “Materials and Methods” and analyzed by SDS-PAGE (Figure 1D). GH11 was slightly better expressed by pPICZ α A than by pGAPZ α A, although at levels definitely lower than those of GH5 and GH45. On the basis of above results, integrative vectors pGAPZ α A and pPICZ were chosen for expressing fungal CAZymes. Two attempts to make DNA constructs easier resulted in negative expression results: the use of Gateway cloning instead of R/L (Additional File 1), and the use of PCR to both generate and linearize integrative expression constructs in a single experiment (Liu et al., 2008; Yu et al., 2012) (Additional File 2). In conclusion, the classical R/L technique followed by linearization by restriction of expression integrative constructs before transformation was used in the rest of this study, as proposed by Invitrogen (easyselect_man.pdf).

Transformation

Frozen Electrocompetent Cells

Invitrogen advises not using frozen electrocompetent cells because freeze/thaw cycles reduce cell transformation efficiency. However, extemporaneously preparing electrocompetent cells is time consuming, which proportionally increases the cost price of recombinant proteins. The protocol used throughout this study and described in Section “Materials and Methods” was kindly provided by one of our collaborators (EIPL) and proved successful with all our expression constructs. No attempt was made to substitute electroporation with chemical transformation because the transformation efficiency was considered too low for the liquid phase transformation described below, although a recently launched chemical compound (BG2) seems promising in this respect (Filyak et al., 2013).

Amount of DNA for Transformation

As mentioned above, highest transformation efficiencies are not required in a recombinant protein expression facility because few clones are sufficient for expressing a recombinant protein. Since the number of individual colonies on agar plates is proportional to the amount of DNA used for transformation for a given transformation efficiency, we assessed the possibility to reduce the amount of DNA used for transformation. Invitrogen protocol suggests using 10 μ g of linear expression construct

per transformation. However, we found that as low as 0.5 μ g of linear expression construct was enough for obtaining the necessary number of transformants for expressing recombinant proteins. Incidentally, using less DNA also allows using plasmid DNA mini-prep and not midi-prep kits, which is both faster and cheaper. An additional gain of time could be to skip the expression construct linearization by restriction, but we found that electroporating yeast cells with 0.5 μ g of non-linearized integrative expression construct failed to give rise to colonies on agar plates (not illustrated). In conclusion, 0.5 μ g of linear expression construct were decided to be, respectively, the default value and DNA state used on the expression platform.

Plating-Free Transformation

Invitrogen’s protocol implies plating cells on Zeocin agar plates after electroporation for selecting transformants. We reasoned that there were no reason why transformants could not be selected in liquid rather than in solid phase (i.e., on agar) provided the selection reagent Zeocin was included in the culture medium. Thereafter, we refer to selection of transformants by plating on Zeocin-containing agar plates as “solid phase” selection, and to selection of transformants by growing in YPD-Zeocin as “liquid phase” selection. The only weakness of the liquid phase option is that transformants are screened as a pool and not as individual clones. As a consequence, the expression screen result is the average of all clones that have been selected by the antibiotic, which can lead to suboptimal expression results.

We compared both approaches to assess whether having individual clones was critical for our project. X33 cells were transformed with linearized pGAPZ α A constructs bearing coding sequences for GH5, GH11, or GH45. After the sorbitol step, 200 μ l of cell suspension in sorbitol were plated and then incubated at 30°C for 3 days as usual, and another 200 μ l of the same cell suspension in sorbitol were used for liquid phase selection as described in Section “Materials and Methods”. When clones began to appear on agar, they were individually used to inoculate 5 ml YPD-Zeocin and then allowed to grow and express the recombinant protein for 4 days. Expression of recombinant proteins of all solid and liquid phase experimental points was assessed by assaying GH5, GH11, and GH45 enzymatic

activities. Results (Figure 2) definitely show that, at least for our three reference proteins, there was no difference between either selection mean. In addition to using less Zeocin than solid phase selection [2 μ l in 2 ml YPD instead of 25 μ l in 25 ml YPD containing 1.2% agar (YPDA; i.e., the volume poured per plate)], liquid phase selection does not preclude individual clones to be selected on plate if necessary since it uses only 200 out of 1000 μ l of the sorbitol suspension of transformed cells and so an additional 200 μ l aliquot can be plated and grown in parallel with the liquid phase selection. In conclusion, the liquid phase selection was chosen as default option for our facility because it allows a yes/no answer to be obtained more quickly than solid state screening. In case of negative or poor response, plated clones can be used as a rescue.

Culture of Transformants

Culture Dish

Based on our experience in recombinant expression screening in *Escherichia coli* (Noguere et al., 2012) and in filamentous fungi (Alberto et al., 2009), the ANSI/SLAS (American National Standards Institute/Society for Laboratory Automation and Screening) format was preferred to classical tubes and flasks for growing cells. This format has two advantages. First, dishes stand alone and do not require additional equipment such as tube or flask holders, which is convenient when many samples have to be processed in parallel as it is often the case on a dedicated platform. Second, it was devised for use with automates, which allows for a direct transfer from culture incubators to robot benchwork.

Although 96-wells deepwell plates have been successfully used for growing and co-expressing recombinant proteins in *P. pastoris* (Geier et al., 2013), 24-wells deepwell plates better fitted with the requirements of this type of platform. By accommodating larger culture volumes than 96-wells deepwell plates (5 instead of 1 ml per well), 24-wells deepwell plates act as a magnifying tool of recombinant protein expression for the following reason. Since recombinant proteins secreted by *P. pastoris* in the culture medium are C-terminally His6-tagged,

they can be concentrated up to about 25 times by collecting them by affinity chromatography on Ni-NTA beads if elution from the affinity column is performed using 200 μ l elution buffer (see further). Hence, even tiny levels of expression can be detected by SDS-PAGE analysis and Coomassie blue staining. By contrast, each well of a 96-wells deepwell plates provides five times less signal on gel for the same expression level because it can accommodate only 1 ml of culture medium. In addition, since oxygen transfer in 24-wells deepwell plates is higher than in 96-wells deepwell plates (Duetz, 2007), one may expect higher expression levels in the former than in the latter. Baffled 16-wells deepwell plates used for growing filamentous fungi (Alberto et al., 2009) were eventually not considered for use on the platform because the number of wells was too small for the intended screening throughput and their geometry unfitted for the automate six needle pipetting arm. In conclusion, 24-wells deepwell plates were chosen as default culture dish.

Managing Zeocin Consumption

Yeast expression plasmids of pGAPZ and pPICZ series confer Zeocin resistance to transformed cells, which allows for easy selection of the latter after plating on Zeocin-containing agar plates. However, Zeocin has two failings: it is expensive and degrades rapidly with time. In particular, it is light sensitive. Therefore, Zeocin-containing agar plates cannot be stored for long periods of time before use and must be protected from light throughout the process ranging from pouring to plating, which makes their handling inconvenient.

We addressed the issues of cost and degradability by testing the possibility of directly adding Zeocin to suspensions of electroporated cells in sorbitol solution rather than to agar plates.

In practice, X33 cells were electroporated with episomal Gateway plasmid pBGP1-DEST bearing GH5 coding sequence. After the sorbitol step, 100 out of 1000 μ l cell suspension were spread on either a Zeocin plate made of 25 ml of YPDA supplemented with 25 μ l of 100 mg/ml Zeocin solution, or on agar plates made of 25 ml of Zeocin-free YPDA. In the latter

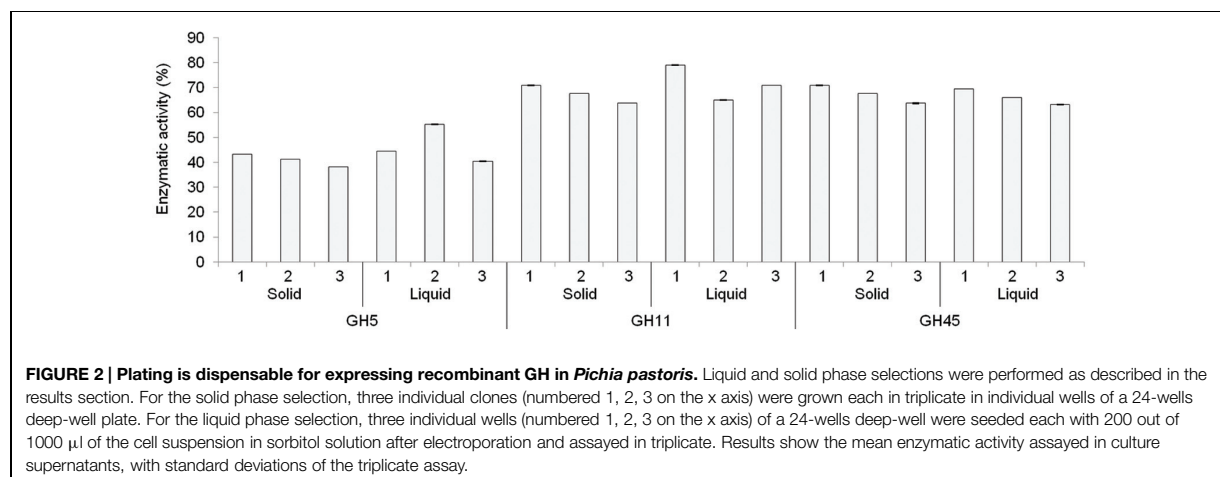
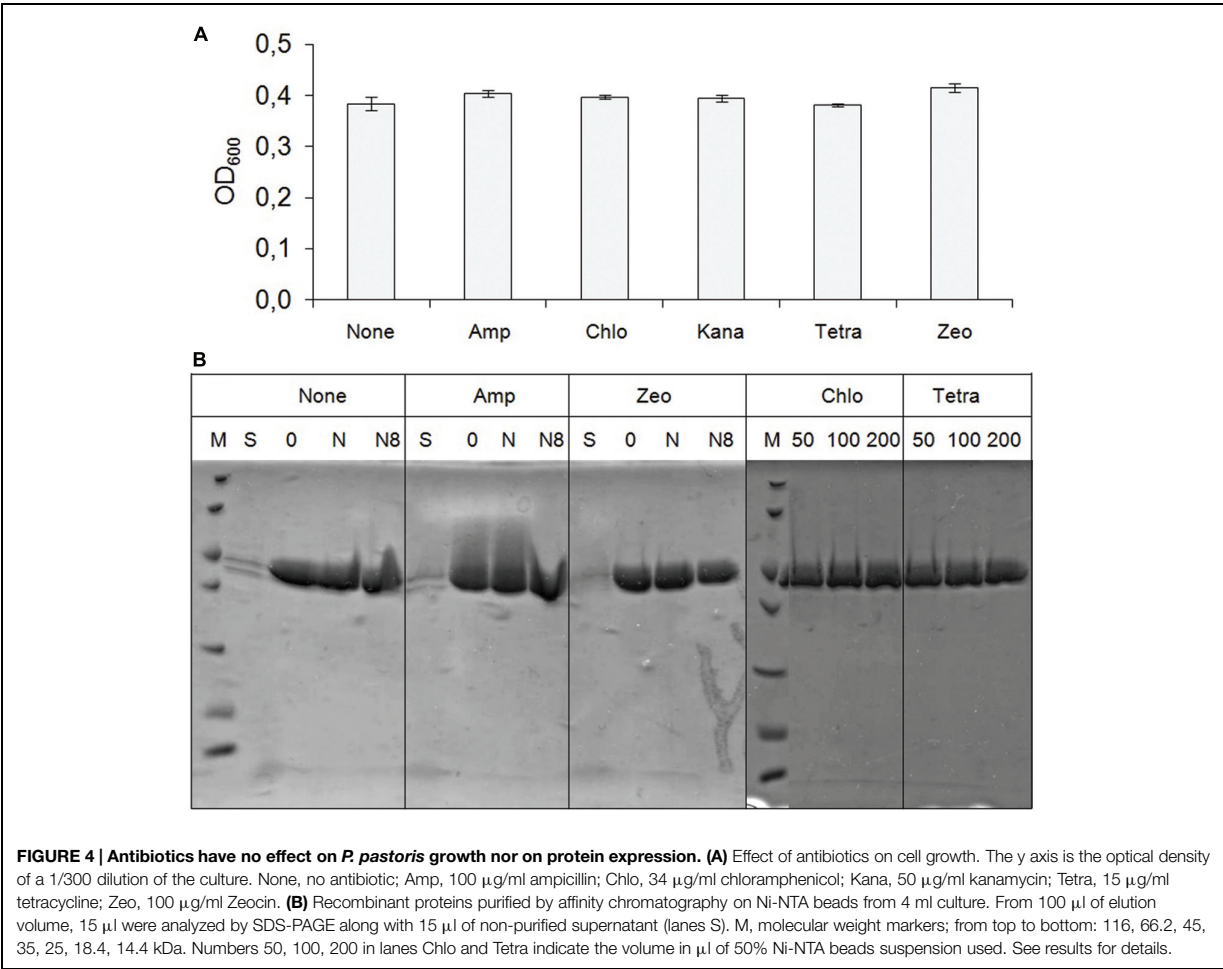
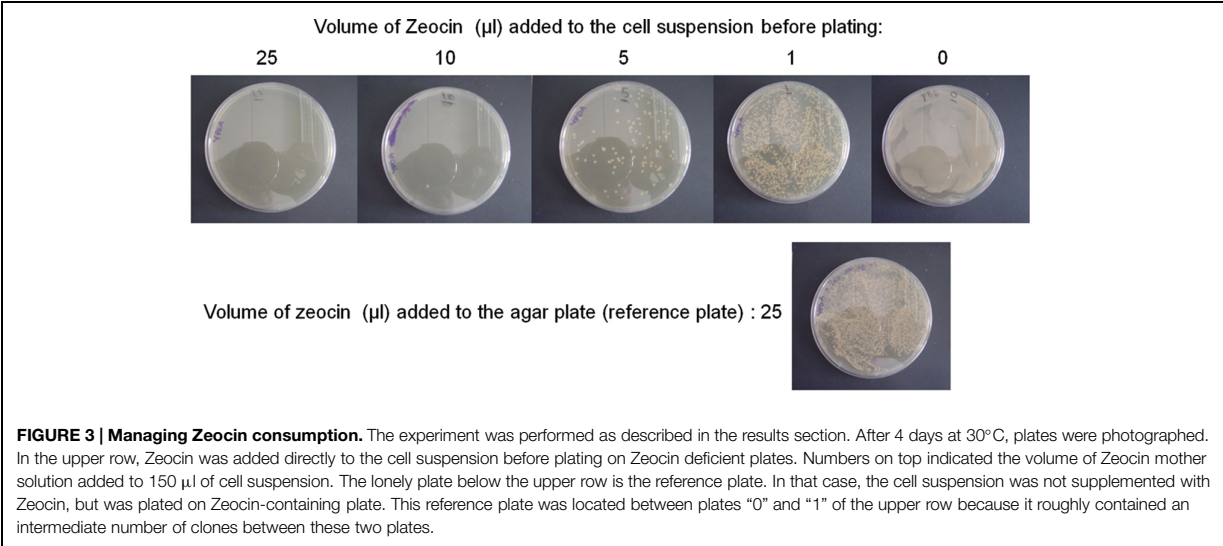
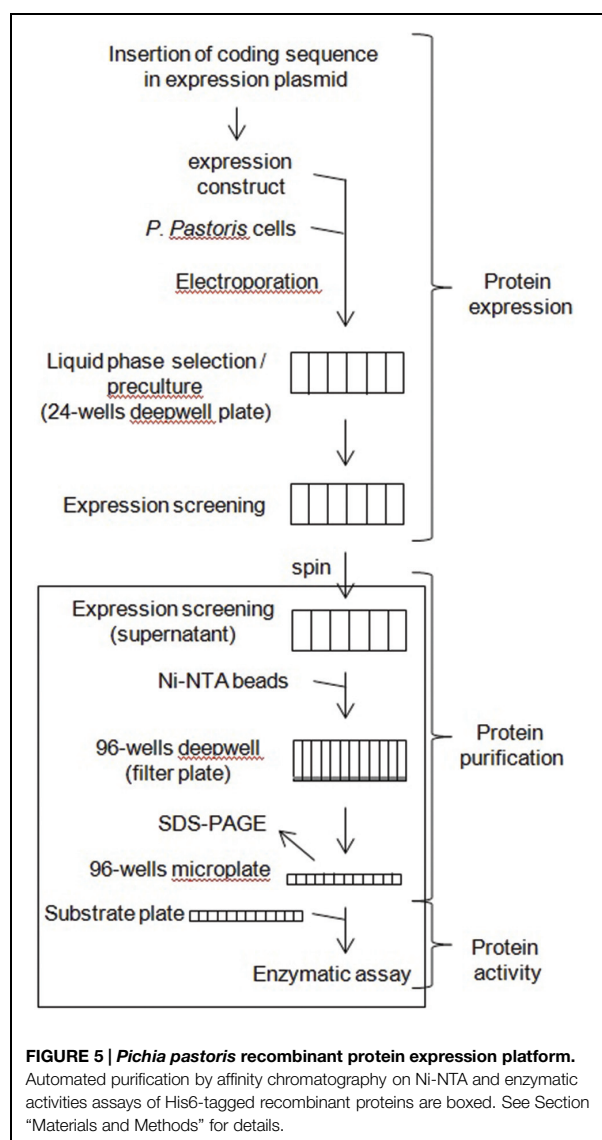


FIGURE 2 | Plating is dispensable for expressing recombinant GH in *Pichia pastoris*. Liquid and solid phase selections were performed as described in the results section. For the solid phase selection, three individual clones (numbered 1, 2, 3 on the x axis) were grown each in triplicate in individual wells of a 24-wells deep-well plate. For the liquid phase selection, three individual wells (numbered 1, 2, 3 on the x axis) of a 24-wells deep-well were seeded each with 200 out of 1000 μ l of the cell suspension in sorbitol solution after electroporation and assayed in triplicate. Results show the mean enzymatic activity assayed in culture supernatants, with standard deviations of the triplicate assay.





case, cell suspensions were supplemented with 0, 1, 5, 10, or 25 μ l of 100 mg/ml Zeocin solution immediately before spreading on Zeocin-free plates. Plates were then incubated at 30°C for 4 days, and the number of colonies was compared. Results (Figure 3) indicate that adding 1 μ l of Zeocin solution directly to the cell suspension just before plating provided roughly the same result as plating the same volume of the same cell suspension on an agar plate supplemented with 25 μ l of Zeocin solution.

Adding Zeocin to the cell suspension immediately before plating rather than in the agar plate allows (i) dividing Zeocin consumption by about 50 for the same selection efficiency, (ii) preparing YPDA plates in advance of use and then storing them without particular caution except asepsis. In conclusion, if the

liquid phase selection described in a previous paragraph cannot be performed because isolating clones is mandatory for some reason, this can be done at lower cost and more conveniently by adding Zeocin to the cell suspension just before plating rather than in the YPDA plate.

Prevention of Bacterial Contamination

Because integrative plasmids are generally used for transforming *P. pastoris* cells for which maintaining antibiotic selection pressure after transformants have been selected is unnecessary, and because the antibiotic used to select transformants (Zeocin) is expensive, no antibiotic is used for growing clones thereafter. Sometimes, however, the absence of antibiotic in rich culture media such as YPD allows bacterial contaminations to occur with the mandatory loss of the contaminated batch. Penicillin and Streptomycin are routinely used to prevent bacterial contaminations in eukaryotic cell line cultures. Being eukaryotes, yeasts should not be sensitive to antibiotics commonly used by microbiologists (ampicillin, chloramphenicol, kanamycin, tetracycline), which could thus be added to the culture medium without deleterious effect while preventing such contaminations.

To check that antibiotics had really no effect on yeast growth, a clone of *P. pastoris* cells expressing GH5 from pGAPZ α A was grown for 4 days in YPD in the presence or absence of the antibiotics listed above at concentrations used in laboratories to select resistant *E. coli*. Biomass was assessed by measuring the optical density of the culture at 600 nm at the end of that period, which definitely demonstrated the total innocuousness of this series of antibiotics (Figure 4A). The amount of recombinant protein secreted in the culture medium was next compared. To that end, His6-tagged GH5 was concentrated by affinity chromatography on Ni-NTA beads under different conditions that are detailed in the next paragraph. Results (Figure 4B) showed no difference between protein expression in the presence or absence of antibiotic, suggesting that antibiotics have no negative effect on the over expression of a recombinant protein in *P. pastoris*. In conclusion, if bacterial contaminations are an issue commonly used antibiotics can provide good protection at much lower cost than Zeocin, and without effect on *P. pastoris* cell growth or protein expression.

Protein Purification

Recombinant proteins were expressed as fusion proteins with an N-terminal signal peptide and a C-terminal His6-tag. This combination has two consequences. First, recombinant proteins are secreted in the culture medium as are biomass-degrading enzymes by filamentous fungi in their native environment. Second, this diluted protein solution can be concentrated up to about 25 times (compare, for example, lanes S to any other lane in Figure 4B) by collecting tagged proteins by affinity chromatography on Ni-NTA beads. The latter has two requirements: high specific binding is favored by slightly alkaline pH (about 8), and low non-specific binding requires high salt concentrations. Since YPD is devoid of any added salt and its pH $\approx$ 6, the addition (Figure 4B) of 0.3 M NaCl with (lanes N8) or without (lanes N) 100 mM Tris pH8 was compared

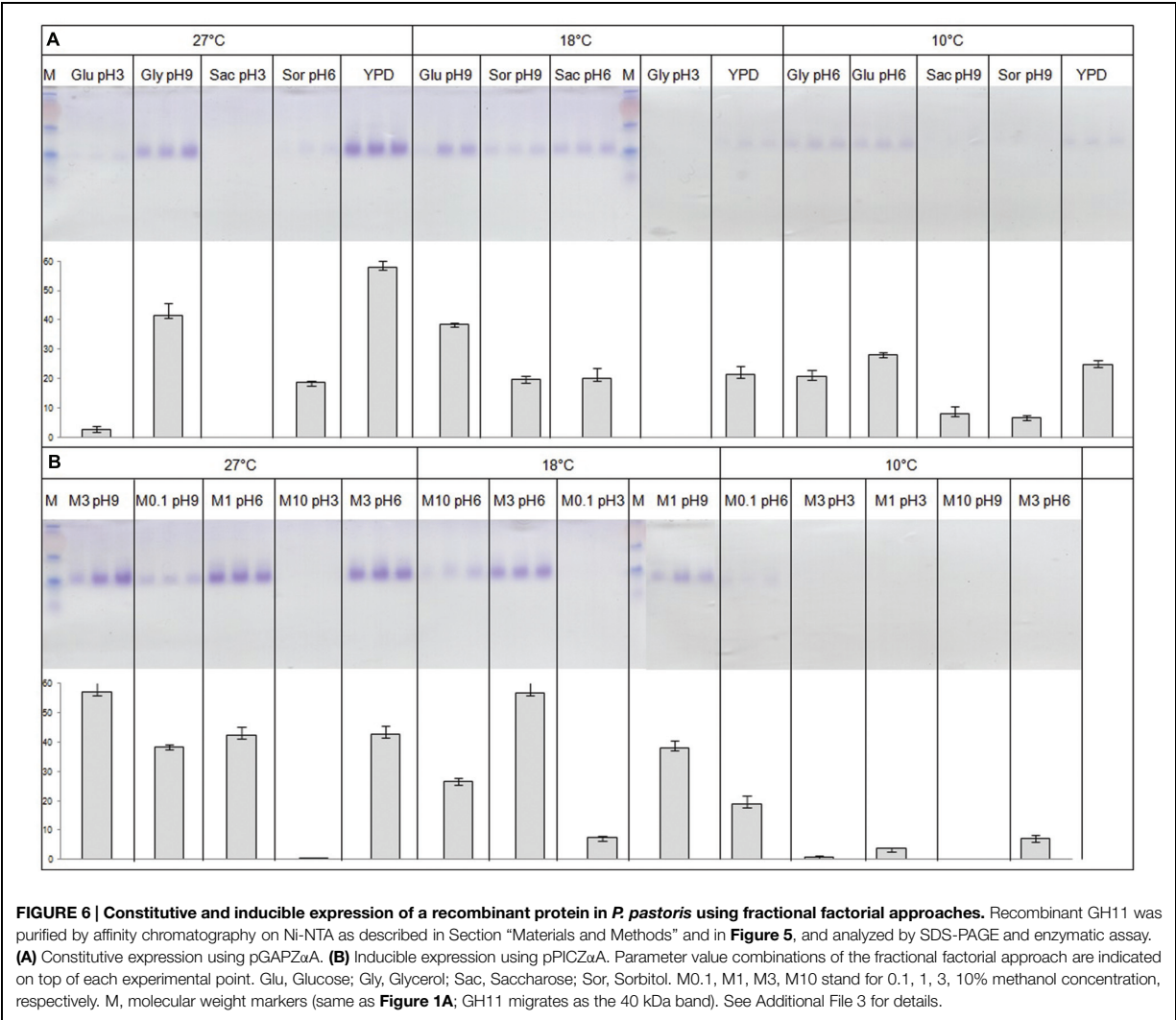
to no addition (lanes 0). Results indicate that such additions were not required. However, when the culture medium pH is willingly maintained acidic (pH3) as in the fractional factorial approach described in the next paragraph, buffering using a final concentration of 100 mM Tris pH8 is mandatory before binding. The amount of Ni-NTA beads proved not limiting since increasing them from 50 to 200 μ l of a 50% beads suspension did not increase the recovery yield (**Figure 4B**, lanes Chlo and Tetra).

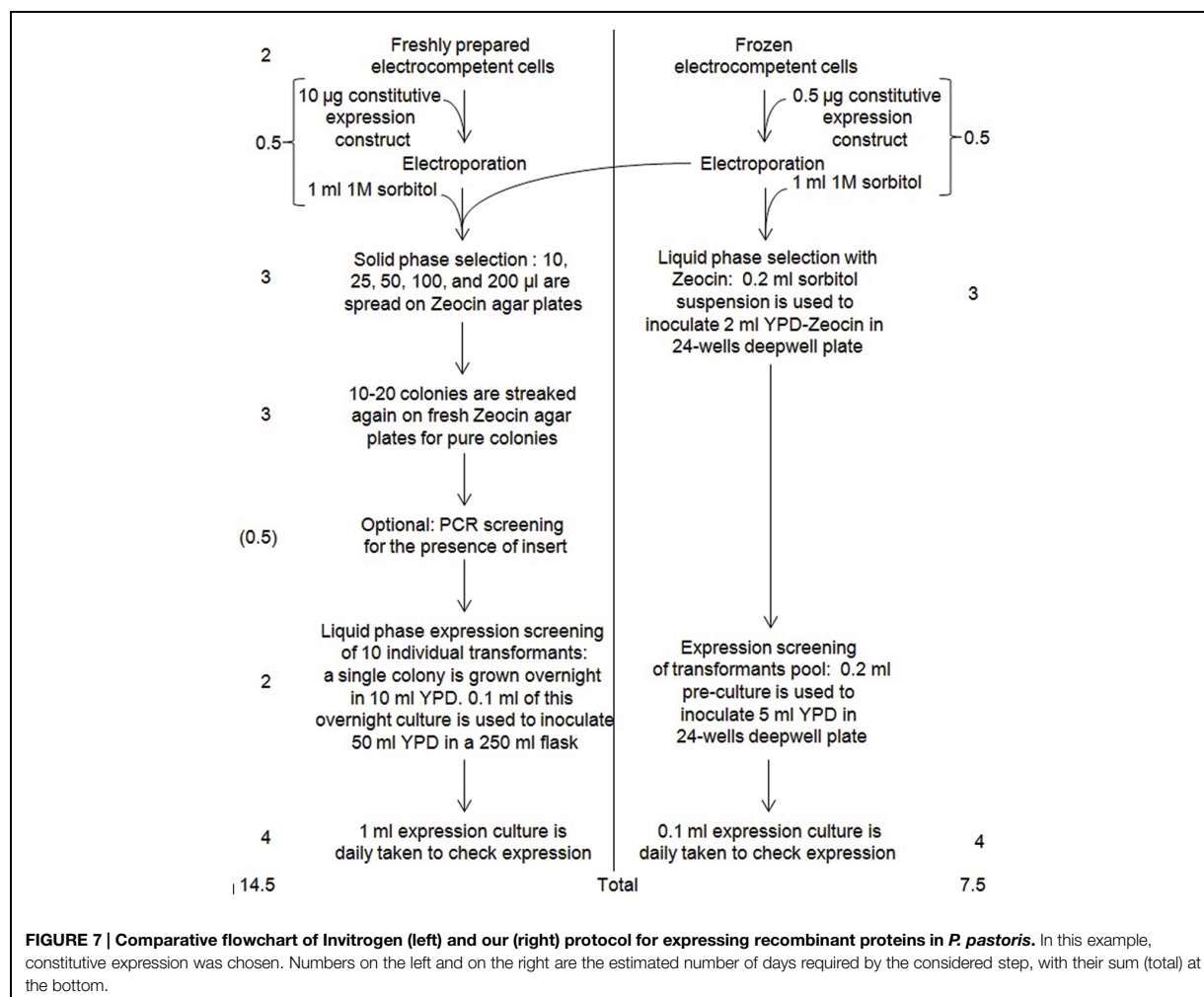
Since deepwell plates can be directly transferred from a shaking incubator to a robot benchwork, the recovery of His6-tagged recombinant proteins by affinity chromatography on Ni-NTA was automated. To that end, Ni-NTA beads were used in deepwell filter plates as described in Section “Materials and Methods.” Note that 100 μ l of beads per well were used because using 50 μ l provided less reproducible results (not illustrated). The flowchart of the automated process is illustrated by **Figure 5**,

and a thorough description of the robot can be found in (Navarro et al., 2010). After elution, recombinant His6-tagged proteins were analyzed by gel electrophoresis using pre-cast gels, and by assaying their enzymatic activity (**Figure 6**).

Expression Conditions

It is our experience that modifying expression parameters in *E. coli* by trial and error is less effective than testing the same parameters in a factorial approach, mainly because parameter interaction is lost in the former case (Benoit et al., 2007; Noguere et al., 2012). Therefore, GH11 expression was tested in two different fractional factorial approaches using pGAPZ α A and pPICZ α A vectors, respectively. A detailed description of the method and of the results is in Additional File 3. In short, both approaches converged to the conclusion that expression was proportional to the temperature, and that an alkaline pH was more favorable than an acidic one (**Figure 6**).





Conclusion

The recombinant protein production facility using *P. pastoris* should avoid using episomal and/or Gateway vectors because the former uselessly request maintaining expensive Zeocin selection throughout the expression process, and the latter failed at efficiently expressing three reference proteins. We have observed that expressing three reference CAZymes was plasmid-dependent. However, since testing both constitutive and inducible expression in parallel might prove impractical for large projects, a serial approach is more realistic. In case of negative result, swapping plasmids is made easy by the identity of cloning regions and reading frames of both vectors. Expression constructs should better be made using classical R/L followed by linearization by restriction. However, large projects may require using synthetic genes with yeast-optimized codons. After electroporation, transformants should be selected with Zeocin in liquid phase as a pool and not as individual colonies on agar plates. However, since electroporated cells can be stored for

weeks at 4°C as sorbitol suspensions and only 200 µl out of 1000 are used for liquid phase selection, another 200 µl aliquot should be plated in parallel to later screen for higher producers (i.e., bearing gene multi-copies) if required. In addition, in case of bacterial contamination it is possible to start a new culture from liquid phase selection pre-culture (i.e., without the need for another electroporation) in the presence of one or more of the anti-microbial antibiotics tested in the present study. For large projects, basic expression conditions (standard culture media, 27°C) are preferred in first intention. By contrast, fractional factorial approaches may be performed in case of single protein project low expression yields and for high-added value proteins. In the ANSI/SLAS format, 24-wells deepwell plates are used for growing yeasts, liquid phase selection of transformants and expression screening. Recombinant proteins secreted in the culture medium are purified and concentrated by automated affinity chromatography on NiNTA beads.

A comparative flowchart of the classical protocol set-up by Invitrogen (pgapz_man.pdf) and of the simplified protocol

TABLE 1 | The different enzymes expressed on the platform are reported.

Fungal strain	CAZy family	CBM	Predicted activity	Genbank ID	cDNA (bp)	Produced	Yield	Reference
<i>Ustilago maydis</i>	GH3	–	β -glucosidase	3634215	2619	No	–	–
<i>Podospira anserina</i>	GH5	–	β -1,4 mannanase	6194921	1236	Yes	>100	Couturier et al., 2011a
<i>P. anserina</i>	GH5	–	β -1,4 mannanase	6187561	1388	No	–	–
<i>P. anserina</i>	GH6	1	Cellobiohydrolase	6187194	1544	Yes	>500	Poidevin et al., 2013
<i>P. anserina</i>	GH6	–	Endoglucanase	6187311	1319	Yes	>500	Poidevin et al., 2013
<i>P. anserina</i>	GH6	1	Cellobiohydrolase	6188027	1258	Yes	>500	Poidevin et al., 2013
<i>P. anserina</i>	GH10	1	β -1,4 xylanase	6188644	1387	No	–	–
<i>P. anserina</i>	GH11	–	β -1,4 xylanase	6197075	742	No	–	–
<i>P. anserina</i>	GH11	1	β -1,4 xylanase	6187330	1060	Yes	<100	Couturier et al., 2011a
<i>P. anserina</i>	GH26	35	β -1,4 mannanase	6188179	1466	Yes	>100	Couturier et al., 2011a
<i>P. anserina</i>	GH27	–	α -galactosidase	6195125	1370	No	–	–
<i>U. maydis</i>	GH27	35	α -galactosidase	3632217	1719	No	–	–
<i>P. anserina</i>	GH43	–	β -xylosidase	6187343	1629	No	–	–
<i>Pichia pastoris</i>	GH45	1	β -1,4 glucanase	8201351	1845	Yes	>500	Couturier et al., 2011b
<i>P. anserina</i>	GH51	–	α -L-arabinofuranosidase	6188578	2251	Yes	>100	Couturier et al., 2011a
<i>U. maydis</i>	GH51	–	α -L-arabinofuranosidase	3628887	2016	Yes	>100	unpublished
<i>U. maydis</i>	GH62	–	α -L-arabinofuranosidase	3632410	996	Yes	>100	Siguiet et al., 2014
<i>P. anserina</i>	GH62	–	α -L-arabinofuranosidase	6188704	1068	Yes	>100	Couturier et al., 2011a; Siguiet et al., 2014
<i>P. anserina</i>	GH67	–	α -glucuronidase	6189855	2591	No	–	–
<i>P. anserina</i>	GH93	–	arabinanase	6188440	1274	Yes	>100	unpublished
<i>P. anserina</i>	GH131	1	β -1,4 glucanase	6187659	1093	Yes	>500	Lafond et al., 2012
<i>Talaromyces funiculosus</i>	CE1	1	Cinnamoyl esterase	AJ291496	1059	Yes	n.d.	unpublished
<i>Penicillium purpurogenum</i>	CE1	1	Acetyl xylan esterase	AF529173	1146	Yes	n.d.	unpublished
<i>Volvariella volvacea</i>	CE1	1	Acetyl xylan esterase	ABI63599	1047	Yes	n.d.	unpublished
<i>Pycnoporus cinnabarinus</i>	CE1	1	Carbohydrate esterase (CE)	CDO72503	1128	Yes	n.d.	unpublished
<i>P. anserina</i>	CE15	1	Methylglucuronic esterase	6187208	1446	Yes	<100	Katsimpouras et al., 2014
<i>P. anserina</i>	CE15	–	Methylglucuronic esterase	6189833	1188	Yes	<100	unpublished
<i>P. anserina</i>	CE16	1	Acetyl esterase	6189799	1329	Yes	<100	unpublished
<i>U. maydis</i>	AA3_2	–	GMC oxidoreductase	3632124	1911	Yes	>500	unpublished
<i>U. maydis</i>	AA3_2	–	Glucose oxidase	3631645	1905	Yes	>100	unpublished
<i>P. anserina</i>	AA3_2	–	Cellobiose dehydrogenase	6188032	2376	Yes	>500	Bennati-Granier et al., 2015
<i>Pycnoporus cinnabarinus</i>	AA3_2	–	Cellobiose dehydrogenase	AF081574	4500	Yes	>500	Bey et al., 2011
<i>U. maydis</i>	AA5_1	–	Glyoxal oxidase	3629211	1959	No	–	–
<i>U. maydis</i>	AA5_1	–	Glyoxal oxidase	3630482	2589	No	–	–
<i>P. anserina</i>	AA9	1	Lytic polysaccharide monooxygenase	6195934	1056	Yes	>100	Bey et al., 2013
<i>P. anserina</i>	AA9	1	Lytic polysaccharide monooxygenase	6192283	1043	Yes	>500	Bey et al., 2013
<i>P. anserina</i>	AA9	–	Lytic polysaccharide monooxygenase	6192256	783	No	–	–
<i>P. anserina</i>	AA9	–	lytic polysaccharide monooxygenase	6190208	1038	Yes	>100	Bennati-Granier et al., 2015
<i>P. anserina</i>	AA9	1	Lytic polysaccharide monooxygenase	6191260	934	Yes	<100	Bennati-Granier et al., 2015
<i>P. anserina</i>	AA9	–	Lytic polysaccharide monooxygenase	6195000	902	Yes	<100	Bennati-Granier et al., 2015
<i>P. anserina</i>	AA9	–	Lytic polysaccharide monooxygenase	6196037	778	Yes	<100	Bennati-Granier et al., 2015
<i>P. anserina</i>	AA9	1	Lytic polysaccharide monooxygenase	6187700	1113	Yes	>100	Bennati-Granier et al., 2015

Yield, amount of secreted recombinant protein (mg/L).

described here for expressing biomass-degrading fungal enzymes is provided in **Figure 7**. Overall, the simplified protocol allows dividing by two the time required to go from transformation to expression screening, and was used as a basis for setting-up a recombinant protein production facility for the heterologous expression of fungal CAZymes in *P. pastoris* (**Figure 5**). **Table 1** summarizes all the results obtained using *P. pastoris* as host for expressing fungal biomass-degrading enzymes in replacement of native or recombinant expression in other fungi such as *A. niger* and *T. reesei* (Levasseur et al., 2004; Poidevin et al., 2009). Switching from the original Invitrogen protocol to the simplified protocol described in this report and now routinely used on the platform has resulted in an increased use of recombinant expression in yeast from a few coding sequences in 2011 to a total of more than 40 today, out of which more than 50% were expressed at high level (> 100 mg/L). The fungal CAZymes functionally produced originate from several filamentous fungi and belong to different CAZy families within the GH, CE, and AA classes. The platform is accessible to the community of CAZyme users upon request.

Author Contributions

MH performed most of the experiments.

SG set up and optimized NiNTA protein purification protocols in collaboration with DN who was more involved in the automation thereof.

AG was in charge of factorial approaches.

References

- Alberto, F., Navarro, D., de Vries, R. P., Asther, M., and Record, E. (2009). Technical advance in fungal biotechnology: development of a miniaturized culture method and an automated high-throughput screening. *Lett. Appl. Microbiol.* 49, 278–282. doi: 10.1111/j.1472-765X.2009.02655.x
- Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006). Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11417–11422. doi: 10.1073/pnas.0604632103
- Bennati-Granier, C., Garajova, S., Champion, C., Grisel, S., Haon, M., Zhou, S., et al. (2015). Substrate specificity and regioselectivity of fungal AA9 lytic polysaccharide monooxygenases secreted by *Podospora anserina*. *Biotechnol. Biofuels* 8, 90. doi: 10.1186/s13068-015-0274-3
- Benoit, I., Coutard, B., Oubelaid, R., Asther, M., and Bignon, C. (2007). Expression in *Escherichia coli*, refolding and crystallization of *Aspergillus niger* feruloyl esterase A using a serial factorial approach. *Protein Expr. Purif.* 55, 166–174. doi: 10.1016/j.pep.2007.04.001
- Bey, M., Berrin, J. G., Poidevin, L., and Sigoillot, J. C. (2011). Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes. *Microb. Cell Fact.* 10, 113. doi: 10.1186/1475-2859-10-113
- Bey, M., Zhou, S., Poidevin, L., Henrissat, B., Coutinho, P. M., Berrin, J. G., et al. (2013). Cello-oligosaccharide oxidation reveals differences between two lytic polysaccharide monooxygenases (family GH61) from *Podospora anserina*. *Appl. Environ. Microbiol.* 79, 488–496. doi: 10.1128/AEM.02942-12
- Couturier, M., Feliu, J., Haon, M., Navarro, D., Lesage-Meessen, L., Coutinho, P. M., et al. (2011a). A thermostable GH45 endoglucanase from yeast: impact of its atypical multimodularity on activity. *Microb. Cell Fact.* 10, 103. doi: 10.1186/1475-2859-10-103

J-GB had the idea of setting-up a recombinant protein expression facility using *P. pastoris* and proposed that CB be in charge of its set-up. He also provided several guidelines and participated in the writing of the manuscript.

CB preliminarily tested different experimental options developed in this study, coordinated the effort of above people and wrote the paper.

Acknowledgments

We are grateful to Dr Frederic Carrière (Enzymology at Interfaces and Physiology of Lipolysis, EIPL, Marseille, France) for the protocol for preparing frozen electro competent cells and for allowing one of us to perform preliminary experiments in his lab where we greatly appreciated the valuable assistance of Justine Baignol. We thank Pr Manabu Arioka at the Laboratory of Microbiology, Department of Biotechnology, the University of Tokyo for providing pBGP1-DEST. This study was funded by the French National Research Agency (ANR FUNLOCK ANR-13-BIME-0002-01).

Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01002>

- Couturier, M., Haon, M., Coutinho, P. M., Henrissat, B., Lesage-Meessen, L., and Berrin, J. G. (2011b). *Podospora anserina* hemicellulases potentiate the *Trichoderma reesei* secretome for saccharification of lignocellulosic biomass. *Appl. Environ. Microbiol.* 77, 237–246. doi: 10.1128/AEM.01761-10
- Cregg, J. M., Cereghino, J. L., Shi, J., and Higgins, D. R. (2000). Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* 16, 23–52. doi: 10.1385/MB:16:1:23
- Damasceno, L. M., Huang, C. J., and Batt, C. A. (2012). Protein secretion in *Pichia pastoris* and advances in protein production. *Appl. Microbiol. Biotechnol.* 93, 31–39. doi: 10.1007/s00253-011-3654-z
- Dashtban, M., Schraft, H., and Qin, W. (2009). Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *Int. J. Biol. Sci.* 5, 578–595. doi: 10.7150/ijbs.5.578
- Duetz, W. A. (2007). Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. *Trends Microbiol.* 15, 469–475. doi: 10.1016/j.tim.2007.09.004
- Filyak, Y., Finiuk, N., Mitina, N., Bilyk, O., Titorenko, V., Hrydzuk, O., et al. (2013). A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. *Biotechniques* 54, 35–43. doi: 10.2144/000113980
- Geier, M., Braun, A., Fladischer, P., Stepniak, P., Rudroff, F., Hametner, C., et al. (2013). Double site saturation mutagenesis of the human cytochrome P450 2D6 results in regioselective steroid hydroxylation. *FEBS J.* 280, 3094–3108. doi: 10.1111/febs.12270
- Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Ottillar, R., et al. (2014). MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Res.* 42, D699–D704. doi: 10.1093/nar/gkt1183
- Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., et al. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 315, 804–807. doi: 10.1126/science.1137016

- Katsimpouras, C., Bénarouche, A., Navarro, D., Karpusas, M., Dimarogona, M., Berrin, J. G., et al. (2014). Enzymatic synthesis of model substrates recognized by glucuronoyl esterases from *Podospira anserina* and *Myceliophthora thermophila*. *Appl. Microbiol. Biotechnol.* 98, 5507–5516. doi: 10.1007/s00253-014-5542-9
- Kittl, R., Kracher, D., Burgstaller, D., Haltrich, D., and Ludwig, R. (2012). Production of four *Neurospora crassa* lytic polysaccharide monooxygenases in *Pichia pastoris* monitored by a fluorimetric assay. *Biotechnol. Biofuels* 5, 79. doi: 10.1186/1754-6834-5-79
- Lafond, M., Navarro, D., Haon, M., Couturier, M., and Berrin, J. G. (2012). Characterization of a broad-specificity β -glucanase acting on β -(1,3)-, β -(1,4)-, and β -(1,6)-glucans that defines a new glycoside hydrolase family. *Appl. Environ. Microbiol.* 78, 8540–8546. doi: 10.1128/AEM.02572-12
- Levasseur, A., Benoit, I., Asther, M., Asther, M., and Record, E. (2004). Homologous expression of the feruloyl esterase B gene from *Aspergillus niger* and characterization of the recombinant enzyme. *Protein Expr. Purif.* 37, 126–133. doi: 10.1016/j.pep.2004.05.019
- Liu, Z., Pscheidt, B., Avi, M., Gaisberger, R., Hartner, F. S., Schuster, C., et al. (2008). Laboratory evolved biocatalysts for stereoselective syntheses of substituted benzaldehyde cyanohydrins. *Chembiochem* 9, 58–61. doi: 10.1002/cbic.200700514
- Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* 42, D490–D495. doi: 10.1093/nar/gkt1178
- Lynd, L., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). How biotech can transform biofuels. *Nat. Biotechnol.* 26, 169–172. doi: 10.1038/nbt0208-169
- Navarro, D., Couturier, M., Damasceno da Silva, G. G., Berrin, J. G., Rouau, X., Asther, M., et al. (2010). Automated assay for screening the enzymatic release of reducing sugars from micronized biomass. *Microb. Cell Fact.* 9, 58. doi: 10.1186/1475-2859-9-58
- Noguere, C., Larsson, A. M., Guyot, J. C., and Bignon, C. (2012). Fractional factorial approach combining 4 *Escherichia coli* strains, 3 culture media, 3 expression temperatures and 5 N-terminal fusion tags for screening the soluble expression of recombinant proteins. *Protein Expr. Purif.* 84, 204–213. doi: 10.1016/j.pep.2012.05.011
- Poidevin, L., Feliu, J., Doan, A., Berrin, J. G., Bey, M., Coutinho, P. M., et al. (2013). Insights into exo- and endoglucanase activities of family 6 glycoside hydrolases from *Podospira anserina*. *Appl. Environ. Microbiol.* 79, 4220–4229. doi: 10.1128/AEM.00327-13
- Poidevin, L., Levasseur, A., Paës, G., Navarro, D., Heiss-Blanquet, S., Asther, M., et al. (2009). Heterologous production of the *Pyromyces equi* cinnamoyl esterase in *Trichoderma reesei* for biotechnological applications. *Lett. Appl. Microbiol.* 49, 673–678. doi: 10.1111/j.1472-765X.2009.02734.x
- Rabert, C., Weinacker, D., Pessoa, A. Jr., and Farias, J. G. (2013). Recombinants proteins for industrial uses: utilization of *Pichia pastoris* expression system. *Braz. J. Microbiol.* 44, 351–356. doi: 10.1590/S1517-83822013005000041
- Sasagawa, T., Matsui, M., Kobayashi, Y., Otagiri, M., Moriya, S., Sakamoto, Y., et al. (2011). High-throughput recombinant gene expression systems in *Pichia pastoris* using newly developed plasmid vectors. *Plasmid* 65, 65–69. doi: 10.1016/j.plasmid.2010.08.004
- Siguer, B., Haon, M., Nahoum, V., Marcellin, M., Burlet-Schiltz, O., Coutinho, P. M., et al. (2014). First structural insights into $\alpha\alpha$ -L-arabinofuranosidases from the two GH62 glycoside hydrolase subfamilies. *J. Biol. Chem.* 289, 5261–5273. doi: 10.1074/jbc.M113.528133
- Sulzenbacher, G., Gruez, A., Roig-Zamboni, V., Spinelli, S., Valencia, C., Pagot, F., et al. (2002). A medium-throughput crystallization approach. *Acta Crystallogr. D Biol. Crystallogr.* 58, 2109–2115. doi: 10.1107/S0907444902013938
- Topakas, E., Moukoulis, M., Dimarogona, M., Vafiadi, C., and Christakopoulos, P. (2010). Functional expression of a thermophilic glucuronoyl esterase from *Sporotrichum thermophile*: identification of the nucleophilic serine. *Appl. Microbiol. Biotechnol.* 87, 1765–1772. doi: 10.1007/s00253-010-2655-7
- Topakas, E., Moukoulis, M., Dimarogona, M., Vafiadi, C., and Christakopoulos, P. (2012). Expression, characterization and structural modelling of a feruloyl esterase from the thermophilic fungus *Myceliophthora thermophila*. *Appl. Microbiol. Biotechnol.* 94, 399–411. doi: 10.1007/s00253-011-3612-9
- Yu, X. W., Wang, R., Zhang, M., Xu, Y., and Xiao, R. (2012). Enhanced thermostability of a *Rhizopus chinensis* lipase by in vivo recombination in *Pichia pastoris*. *Microb. Cell Fact.* 11, 102. doi: 10.1186/1475-2859-11-102

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Haon, Grisel, Navarro, Gruet, Berrin and Bignon. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Ex. 1031 Krainer 2015



TECHNICAL NOTES

Open Access

Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*

Florian W Krainer^{1†}, Simona Capone^{2†}, Martin Jäger², Thomas Vogl¹, Michaela Gerstmann¹, Anton Glieder¹, Christoph Herwig² and Oliver Spadiut^{2*}

Abstract

Background: Insufficient incorporation of heme is considered a central impeding cause in the recombinant production of active heme proteins. Currently, two approaches are commonly taken to overcome this bottleneck; metabolic engineering of the heme biosynthesis pathway in the host organism to enhance intracellular heme production, and supplementation of the growth medium with the desired cofactor or precursors thereof to allow saturation of recombinantly produced apo-forms of the target protein. In this study, we investigated the effect of both, pathway engineering and medium supplementation, to optimize the recombinant production of the heme protein horseradish peroxidase in the yeast *Pichia pastoris*.

Results: In contrast to studies with other hosts, co-overexpression of genes of the endogenous heme biosynthesis pathway did not improve the recombinant production of active heme protein. However, medium supplementation with hemin proved to be an efficient strategy to increase the yield of active enzyme, whereas supplementation with the commonly used precursor 5-aminolevulinic acid did not affect target protein yield.

Conclusions: The yield of active recombinant heme peroxidase from *P. pastoris* can be easily enhanced by supplementation of the cultivation medium with hemin. Thereby, secreted apo-species of the target protein are effectively saturated with cofactor, maximizing the yield of target enzyme activity.

Keywords: *Pichia pastoris*, Recombinant protein production, Plant peroxidase, Horseradish peroxidase, Metabolic engineering, Cofactor, Heme, Heme biosynthesis, Apo-protein

Background

The methylotrophic yeast *Pichia pastoris* is a valuable host for the recombinant production of complex proteins. A considerable number of these proteins requires cofactors, amongst others heme, to form active biocatalysts. Heme biosynthesis (Figure 1) is tightly regulated and highly conserved throughout evolution [1-3].

To deepen our understanding on pathway regulation and improve cofactor availability for recombinant heme proteins, metabolic engineering of the heme biosynthesis pathway has been performed in *Aspergillus niger* [4,5], *Escherichia coli* [6] and *Saccharomyces cerevisiae* [7-9]. Despite the high conservation of the heme biosynthesis

pathway, distinct differences were found among the different species. In *E. coli*, formation of 5-aminolevulinic acid (ALA) by the *HEM1*-encoded ALA synthase was described as rate-limiting step [10], whereas in *S. cerevisiae* the *HEM2*- and *HEM3*-encoded porphobilinogen synthase and deaminase, respectively, were described as rate-limiting [7]. Based thereon, overexpression of *HEM3* alone or in combination with *HEM2* and *HEM12* was described to be a valuable strategy to augment the production of recombinant heme proteins in *S. cerevisiae* [9,11]. Despite these promising results, there are some potential pitfalls connected to metabolic pathway engineering, such as the additional metabolic burden upon overexpression of multiple genes as well as an accumulation of free intracellular porphyrin intermediates which can lead to the formation of reactive oxygen species [12]. Thus, medium supplementation with iron, heme precursors or hemin (the ferric chloride species of heme) was assessed as alternative

* Correspondence: oliver.spadiut@tuwien.ac.at

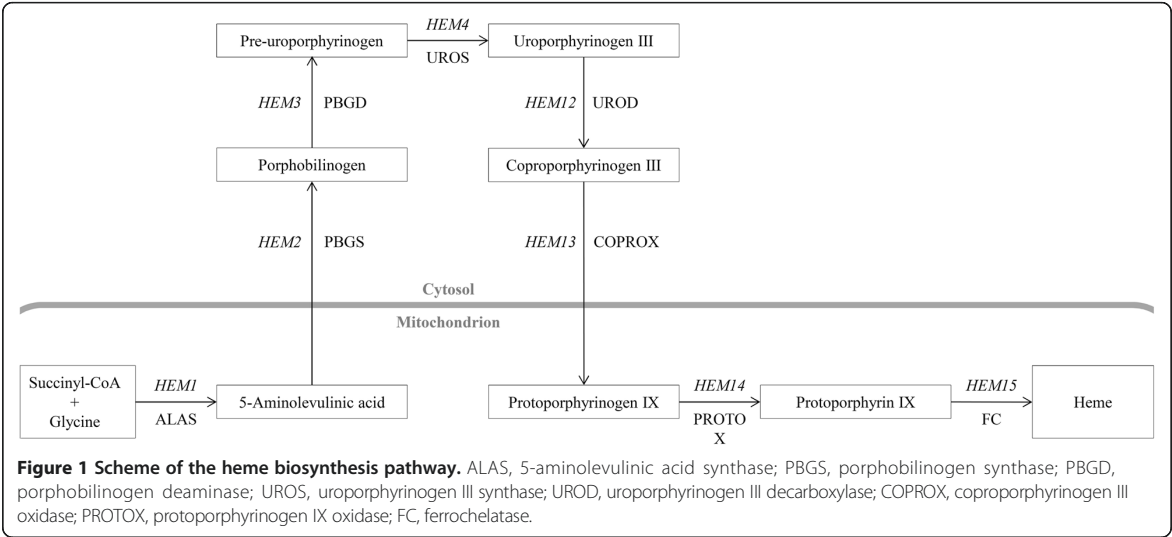
†Equal contributors

²Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, 1060 Vienna, Austria

Full list of author information is available at the end of the article



© 2015 Krainer et al.; licensee BioMed Central. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.



strategy to effectively saturate recombinant apo-species of heme proteins [13-17].

In this study, we aimed at optimizing heme availability and thus boost the amount of recombinant active heme protein, namely horseradish peroxidase (HRP), in the yeast *P. pastoris* by evaluating pathway engineering and medium supplementation. In a systematic approach, we co-overexpressed HRP with genes of the endogenous heme biosynthesis pathway of *P. pastoris*, and assessed the effect of medium supplementation with iron, ALA and hemin on the yield of target enzyme activity.

Results and discussion

Heme biosynthesis pathway in *Pichia pastoris*

The heme biosynthesis pathway of several organisms has been described before and was found to be highly conserved [1-3]. Based on the *HEM* gene sequences from *S. cerevisiae*, we identified the corresponding homologs

in the partially annotated genome of *P. pastoris* [18] *in silico* (Table 1). Sanger sequencing confirmed the correct nucleotide sequences of *HEM1*, *HEM2*, *HEM3*, *HEM4*, *HEM12*, *HEM13* and *HEM14*. For *HEM15*, nucleotide 918 was G in the GenBank database entries of the published genomes of *P. pastoris* strains CBS 7435 and GS155, but T in the Sanger-sequenced CBS 7435 *P. pastoris* strain used in the present study (Additional file 1). However, this single nucleotide polymorphism only resulted in a silent mutation and did not alter the amino acid sequence of the encoded protoheme ferro-lyase.

Co-overexpression of *HEM* genes in microscale cultivations

In a recent study it was shown that metabolic engineering of the heme biosynthesis pathway allowed higher yields of active recombinant heme protein in *S. cerevisiae* [11]. Thus, we co-overexpressed either of the eight involved

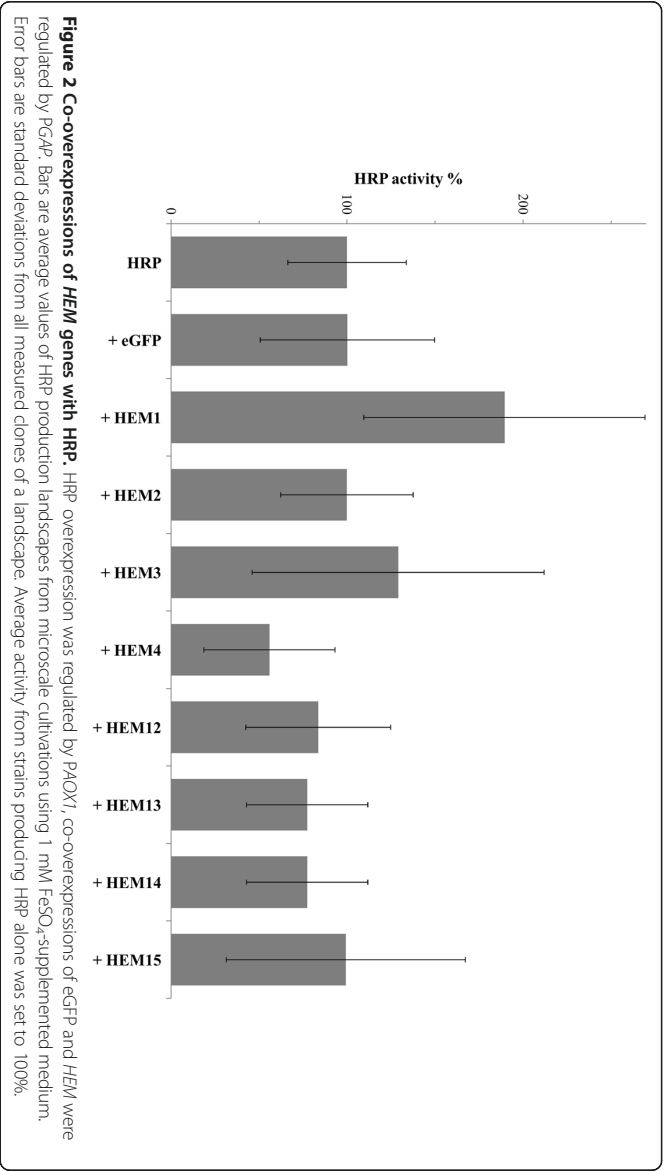
Table 1 *In silico* identification of *HEM* homologs in *P. pastoris*

<i>S. cerevisiae</i>	<i>P. pastoris</i>			Sequence identity [%]
Gene annotation	Gene annotation	Chromosome	COG annotation	
<i>HEM1</i>	<i>HEM1</i>	II	5-aminolevulinate synthase	67.7
<i>HEM2</i>	<i>HEM2</i>	IV	delta-aminolevulinic acid dehydratase	75.3
<i>HEM3</i>	<i>HEM3</i>	I	porphobilinogen deaminase	54.0
<i>HEM4</i>	<i>HEM4</i>	II	uroporphyrinogen-III synthase	42.3
<i>HEM12</i>	n/a	III	uroporphyrinogen decarboxylase	73.2
<i>HEM13</i>	<i>HEM13</i>	III	coproporphyrinogen III oxidase	65.5
<i>HEM14</i>	n/a	IV	protoporphyrinogen oxidase	33.7
<i>HEM15</i>	n/a	III	protoheme ferro-lyase (ferrochelatase)	61.2

Genes *HEM12*, *HEM14* and *HEM15* were not annotated (n/a) for *P. pastoris* CBS 7435. Their chromosomal position in the genome, eukaryotic cluster of orthologous groups (COG; [19]) annotations and identities of the encoded amino acid sequences from *S. cerevisiae* and *P. pastoris* are shown.

HEM genes (Table 1) using the strong constitutive promoter *PGAP* [20] in a *P. pastoris* strain recombinantly producing the heme protein HRP. The screenings revealed trends of co-overexpressed *HEM1* and *HEM3* to be potentially beneficial for the production of active HRP (Figure 2). Surprisingly, there also seemed to be a negative trend upon co-overexpression of *HEM4*. We hypothesize, that *HEM4* co-overexpression might have led to the accumulation of a cytotoxic intermediate, which increased intracellular stress and ultimately caused a disadvantageous production environment for HRP under the tested conditions. At this point, we did not follow up on the effects of *HEM4* co-overexpression. Since co-overexpression of eGFP as negative control did not affect HRP productivity, the observed activity-enhancing trends seen for *HEM1* and *HEM3* were considered a consequence of the co-overexpressed *HEM* gene, although the standard deviation in these experiments was high. The high standard deviations in this screening (Figure 2) were caused by the transformant-to-transformant variation typically observed for *P. pastoris* [21]. Even the transformation of a single gene results in transformants showing different expression strengths. The majority of the transformants shaping the landscape behaved similar but a few transformants showed either no expression or elevated levels, leading to high standard deviations. Supplementation of the cultivation medium with 1 mM FeSO₄ to alleviate a potential iron limitation gave comparable results to non-supplemented medium

and did not affect the observed trends upon *HEM* gene co-overexpression (data not shown). In addition to strong constitutive co-overexpression of either *HEM1* or *HEM3* from *PGAP*, we also tested co-overexpression of these two genes from either *PAOX1* or *PCAT1*. Both promoters are strongly methanol-inducible, however *PCAT1* is already active in the absence of glucose or glycerol and then even further induced by addition of methanol, thereby allowing *HEM* co-overexpression already prior to, but also during HRP production [Vogl *et al.*, manuscript in preparation]. In order to indirectly assess the general functionality and applicability of the employed co-overexpression construct, we measured fluorescence of the eGFP co-overexpression control transformants and found this co-overexpression partner to be successfully produced (Additional file 2). Thus, we considered the endogenous *HEM* co-overexpression partners to be co-overexpressed in a comparable fashion. Ultimately, *PAOX1*-regulated co-overexpressions of *HEM1* and *HEM3* with HRP were found most promising (Additional file 3) and the best performing strains were used for further characterizations. Since the data from microscale cultivations indicated merely trends for potential beneficial effects of co-overexpressed *HEM1* or *HEM3*, we aimed at obtaining more reliable data from controlled cultivations of the best performing strains in bioreactors. Prior to bioreactor cultivations, the strain producing HRP alone (hereafter called benchmark



strain) as well as the *HEM1* and *HEM3* co-overexpressing strains (hereafter called *HEM1* and *HEM3* strains) were characterized by quantitative real-time PCR as strains with single copy integration of the HRP gene. Since these strains contained the same HRP gene dosage, genomic rearrangement of HRP gene copy number upon transformation of a *HEM* co-overexpression construct was excluded and the strains were regarded comparable in terms of target gene dosage.

Co-overexpression of *HEM* genes in bioreactor cultivations

For physiological strain characterisation of the three yeast strains we employed a previously reported strategy of dynamic batch cultivations with methanol pulses [22-25]. The corresponding off-gas signals, specific uptake rates and yields are shown in Additional file 4. Physiological strain characteristic parameters of the three strains are summarized in Table 2. As shown in Table 2, C-balances for all cultivations closed to 1.0 indicating solid data quality. Apparently, the yeast strains were physiologically not impaired by co-overexpression of either *HEM1* or *HEM3* since adaptation time to methanol, yields and specific substrate uptake rates were comparable. However, co-overexpressing these genes did not boost the production of active HRP either. Whereas the benchmark and the *HEM1* strain showed comparable production titres, the *HEM3* strain even showed a 50% lower specific and volumetric productivity than the benchmark strain. Taken together, it turned out that the trends seen in microscale data could not be seen in the bioreactor. A possible explanation for these diverging findings could be found in the considerably different cultivation conditions. Microscale cultivations may challenge cell growth and productivity by phases of O₂ shortage or starvation, whereas a bioreactor provides optimal cultivation conditions.

Considering the complex and so far poorly understood regulation of heme biosynthesis, a multitude of single or combined factors may have either positive or negative effects on this pathway. Thus, additional studies will be required to unravel the regulation of heme biosynthesis, in order to allow non-speculative conclusions. Since our selection of *HEM* co-overexpression partners was based on trends from microscale cultivations, we cannot exclude,

that either of the remaining six *HEM* genes or combinations thereof might yield more beneficial results than co-overexpressed *HEM1* or *HEM3* in bioreactor cultivations. Ultimately, in contrast to analogous studies in *S. cerevisiae* [9,11], metabolic pathway engineering in *P. pastoris* did not prove to be a useful strategy to allow higher titres of recombinant active heme protein, suggesting distinct differences of this pathway between the two yeasts. However, co-overexpression of two or more *HEM* genes from a library of promoters of varying strengths might still enhance endogenous heme biosynthesis in *P. pastoris* and will be assessed in future studies.

Medium supplementation

Medium supplementation in microscale cultivations

We tested ALA, FeSO₄ and hemin as medium supplements in microscale cultivations of the benchmark strain. Supplementation with ALA at an excess concentration of 1 mM, as reported in literature [22,26,27], did not result in measurably more active product. However, addition of 1 mM FeSO₄ boosted the amount of active HRP up to 7-fold (Figure 3), hinting at a potential shortage of iron in heme biosynthesis. Upon supplementation of the minimal medium with different concentrations of hemin, we even measured 18-fold increased HRP activity compared to non-supplemented conditions. We also investigated the effect in case both, FeSO₄ and hemin, were supplemented concomitantly and observed that the beneficial effect of FeSO₄ became less pronounced with increasing concentrations of hemin (Figure 3). Apparently, as all apo-HRP was readily saturated with cofactor at a hemin concentration of 10 μM, additional excess of iron did not improve HRP activities any further and the cofactor bottleneck was opened up.

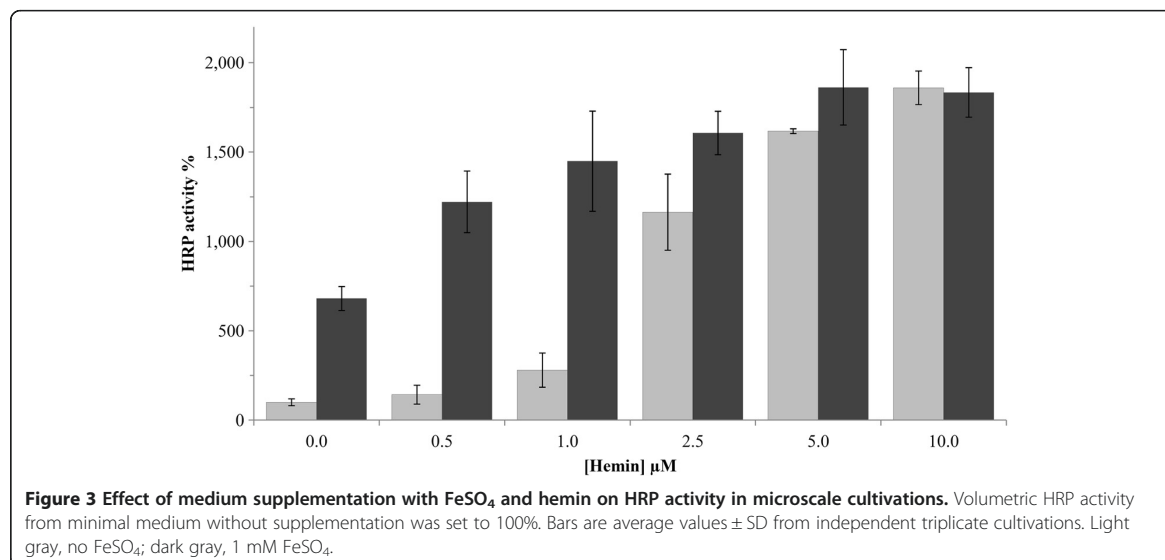
ALA supplementation in the bioreactor

We performed a comparative cultivation experiment in the bioreactor to confirm the results obtained in microscale cultivations that the commonly used supplement ALA [22,26,27] does not affect the production of a recombinant active heme protein in *P. pastoris*. We cultivated the benchmark strain in parallel dynamic batch cultivations with and without the presence of 1 mM ALA. Table 3 shows that both, strain physiology and productivity, were

Table 2 Strain characteristic physiological parameters determined for the benchmark, *HEM1* and *HEM3* strain

Strain	$\Delta\text{time}_{\text{adapt}}$ [h]	$Y_{X/\text{MeOH}}$ [C-mol/C-mol]	$Y_{\text{CO}_2/\text{MeOH}}$ [C-mol/C-mol]	C-balance	q_{MeOH} [mmol/g/h]	q_p [U/g/h]	r_p [U/L/h]
Benchmark	8.2	0.02	1.00	1.02	0.75	1.11	37.5
<i>HEM1</i>	8.2	0.04	0.94	0.98	0.69	1.10	35.9
<i>HEM3</i>	7.9	0.03	0.93	0.96	0.70	0.49	17.1

$\Delta\text{time}_{\text{adapt}}$, time for adaptation of the culture to methanol; $Y_{X/\text{MeOH}}$, $Y_{\text{CO}_2/\text{MeOH}}$, yields of biomass or CO₂ per C-mol of substrate methanol; C-balance, sum of $Y_{X/\text{MeOH}}$ and $Y_{\text{CO}_2/\text{MeOH}}$ which ideally should result in 1.0; q_{MeOH} , average specific uptake rate of methanol during consecutive methanol pulses; q_p , specific HRP productivity; r_p , volumetric HRP productivity calculated from the point of induction until the end of cultivation.



not affected by the presence of ALA. Since the amount of active HRP was the same for both conditions, we concluded that ALA was not beneficial for the activation of the produced apo-HRP. This observation is different from what was described previously by Morawski *et al.*, where a 32% increase in HRP activity was measured in the culture supernatant after supplementation with ALA, trace elements and thiamine [17]. Thus, we speculate that the activation effect observed by Morawski *et al.* was rather caused by the addition of the iron-containing trace element solution than by ALA. Furthermore, our results conclusively showed that the intracellular production of ALA in the heme biosynthesis pathway of *P. pastoris* was not the rate limiting step in contrast to *E. coli* [10], which is also in agreement with the study of Arrese *et al.* [28].

Optimization of hemin supplementation in the bioreactor

Based on the results obtained in microscale cultivations, we supplemented the cultivation broth of three parallel dynamic batch cultivations of the benchmark strain with 0.1, 1.0 or 10.0 μM hemin. As shown in Table 4, adaptation time, yields and uptake rates of the benchmark

strain were not affected by the presence of hemin. In terms of productivity, both the specific and the volumetric productivity were doubled at a concentration of 10.0 μM hemin. Since the total extracellular protein content was comparable in all three cultivations, we concluded that not the productivity of the yeast was altered, but rather that the produced apo-protein was activated posttranslationally by hemin in the cultivation broth. Based on our previous experiences with this expression system [22,23,29,30], we estimated the amount of HRP in the cultivation broth to be $< 3 \mu\text{M}$. Thus, we concluded that an excess hemin concentration is required to effectively saturate secreted apo-HRP (Table 4).

Activation studies with hemin

To prove our hypothesis of a posttranslational conversion of apo-HRP to holo-HRP by hemin, we incubated cell-free, sterile-filtered supernatant from a non-supplemented benchmark cultivation with different concentrations of hemin and analyzed activity over time. As shown in Table 5, already after 5 min of incubation a concentration dependent activation was observed and after 72 h of incubation the initial activity was even doubled. Based thereon,

Table 3 Strain specific physiological parameters of the benchmark strain with and without ALA supplementation

ALA [1 mM]	$\Delta\text{time}_{\text{adapt}}$ [h]	$Y_{X/\text{MeOH}}$ [C-mol/C-mol]	$Y_{\text{CO}_2/\text{MeOH}}$ [C-mol/C-mol]	C-balance	q_{MeOH} [mmol/g/h]	q_p [U/g/h]	r_p [U/L/h]
+	8.1	0.03	0.95	0.98	0.73	1.08	36.7
-	8.2	0.04	0.94	0.98	0.76	1.09	37.2

$\Delta\text{time}_{\text{adapt}}$, time for adaptation of the culture to methanol; $Y_{X/\text{MeOH}}$, $Y_{\text{CO}_2/\text{MeOH}}$, yields of biomass or CO_2 per C-mol of substrate methanol; C-balance, sum of $Y_{X/\text{MeOH}}$ and $Y_{\text{CO}_2/\text{MeOH}}$ which ideally should result in 1.0; q_{MeOH} , average specific uptake rate of methanol during consecutive methanol pulses; q_p , specific HRP productivity; r_p , volumetric HRP productivity calculated from the point of induction until the end of cultivation.

Table 4 Strain characteristic physiological parameters determined for the benchmark strain cultivated in hemin-supplemented media

Hemin [μM]	Δtime _{adapt} [h]	Y _{X/MeOH} [C-mol/C-mol]	Y _{CO₂/MeOH} [C-mol/C-mol]	C-balance	q _{MeOH} [mmol/g/h]	q _p [U/g/h]	r _p [U/L/h]
0.1	6.5	0.04	0.89	0.93	0.69	1.10	34.3
1.0	6.4	0.04	0.93	0.97	0.71	1.26	36.8
10.0	6.5	0.03	0.89	0.96	0.70	2.35	73.2

Δtime_{adapt}, time for adaptation of the culture to methanol; Y_{X/MeOH}, Y_{CO₂/MeOH}, yields of biomass or CO₂ per C-mol of substrate methanol; C-balance, sum of Y_{X/MeOH} and Y_{CO₂/MeOH} which ideally should result in 1.0; q_{MeOH}, average specific uptake rate of methanol during consecutive methanol pulses; q_p, specific HRP productivity; r_p, volumetric HRP productivity.

we concluded that hemin should be present in excess to allow effective apo-protein activation.

Conclusions

In this study we present a systematic approach to optimize heme availability and thus boost the amount of active heme protein produced in the yeast *P. pastoris* by evaluating metabolic pathway engineering and medium supplementation. The results can be summarized as:

- The heme biosynthesis pathway of *P. pastoris* was analyzed and corresponding genes were identified and annotated *in silico*.
- In contrast to previous studies, overexpression of single *HEM* genes did not result in enhanced activity or higher yield of the model heme protein HRP in bioreactor cultivations of recombinant *P. pastoris* strains. However, combinations of *HEM* genes co-overexpressed from a library of differently regulated promoters might still enhance endogenous heme biosynthesis of *P. pastoris*.
- Medium supplementation with the traditionally used and pricy heme precursor ALA did not increase the yield of active product and can be omitted in future cultivations. FeSO₄ and hemin on the other hand turned out to be useful medium supplements to increase the yield of active heme protein.
- Hemin was identified as the most effective supplement. It activated the secreted model heme protein posttranslationally in the cultivation broth and should be added in moderate excess to effectively saturate secreted apo-species of the target protein.

Table 5 Posttranslational activation of apo-HRP with hemin

Hemin [μM]	Specific activity [U/mg] after 5 min of incubation	Specific activity [U/mg] after 72 h of incubation
-	34.8	35.3
1.0	42.4	67.0
5.0	46.8	75.4
10.0	51.8	77.8

Cell-free cultivation supernatant was incubated with the indicated concentrations of hemin and volumetric HRP activity was measured after 5 min and 72 h.

The results shown in this study present a guideline for the successful recombinant production of active heme protein in the yeast *P. pastoris* and offer an easy-to-do solution to maximize the ratio of holo- over apo-protein resulting in a considerable increase of active target protein.

Methods

Chemicals

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), D-(+)-biotin and hemin were purchased from Sigma-Aldrich (Austria). Difco™ yeast nitrogen base (YNB) w/o amino acids, Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson (Austria). Zeocin™ was obtained from InvivoGen (France). Other chemicals were obtained from Carl Roth (Germany).

Pichia pastoris strains

All strains in this study were based on the *P. pastoris* wildtype strain CBS 7435 (identical to NRRL Y-11430 and ATCC 76273). The Mut^S phenotype has been found previously to be superior over the Mut⁺ phenotype for recombinant HRP production in terms of volumetric productivity and production efficiency [24]. Hence, an *aox1* deletion strain (*PpMutS*) was used as starting strain [31]. Homologs of the *HEM* genes of *S. cerevisiae* were identified by BLAST searches [32] in the published genome sequence of *P. pastoris* CBS 7435 [18] and the sequences were verified by Sanger sequencing (Microsynth, Austria). Sequence identities of the *HEM* gene products from *S. cerevisiae* and *P. pastoris* were determined on the LALIGN server.

The HRP expression construct was based on the pPpT4_α_S vector [31] where HRP C1A expression was under control of the promoter of the *AOX1* gene (*PAOX1*). Transformants were identified by Zeocin™ resistance. The *S. cerevisiae* MATα prepro signal peptide facilitated HRP secretion. All *HEM* co-overexpression constructs were based on the plasmid pPpKan_S [32], harboring a kanamycin resistance gene for selection and the strongly methanol-inducible *PAOX1* for gene expression. Also, the promoters *PGAP* and *PCAT1* [Vogl et al., manuscript in preparation] were tested for co-overexpression. Transformation of the HRP expression cassette to *PpMutS* and

of the co-overexpression constructs to the *PpMutS*-based HRP production strain (called benchmark strain) was performed as described previously [24]. All eight *HEM* genes of the heme biosynthesis pathway were co-overexpressed separately and their influence on HRP production was studied by cultivation of approximately 80 transformants per co-overexpression construct in 96-deep well plates (DWP). As negative co-overexpression control, eGFP was co-overexpressed with HRP. The copy number of the HRP gene in selected strains was determined by quantitative real-time PCR according to a previous protocol [33] and as described recently [24].

Microscale cultivations in 96-deep well plates

Microscale cultivations in 96-DWPs were performed similar to [34]: Strains were grown in 250 μ L BMD1% (11 g/L α -D(+)-glucose monohydrate, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) at 28°C, 320 rpm, 80% humidity. After approximately 60 h, an induction pulse of 250 μ L BMM2 (1% (v/v) methanol, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) was added, followed by three pulses of 50 μ L BMM10 (5% (v/v) methanol, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) per well 12, 24 and 36 h after the first pulse. HRP activity was determined by mixing 15 μ L supernatant with 140 μ L assay solution (1 mM ABTS, 0.9 mM H₂O₂, 50 mM sodium acetate, pH 4.5) in a microtiter plate and following the increase in absorbance at 405 nm on a Spectramax Plus 384 platereader (MolecularDevices, Germany) at room temperature. Medium supplementation studies were performed by additions of ALA, FeSO₄ and hemin to BMD1% from a 100 mM ALA stock, a 100 mM FeSO₄ stock and a 500 μ M hemin stock (in 10 mM KOH), respectively.

Bioreactor cultivations

Preculture

Precultures were grown in YNB medium (0.1 M potassium phosphate buffer, pH 6.0; 3.4 g/L YNB w/o amino acids and ammonium sulfate; 10 g/L (NH₄)₂SO₄; 400 mg/L biotin; 20 g/L glucose) in 1 L shake flasks at 30°C and 230 rpm for 24 h.

Dynamic batch cultivations

For dynamic batch cultivations, 1.8 L of double concentrated basal salt medium (BSM; 26.7 mL/L 85% phosphoric acid; 1.17 g/L CaSO₄·2H₂O; 18.2 g/L K₂SO₄; 14.9 g/L MgSO₄·7H₂O; 4.13 g/L KOH; 0.3 mL/L Antifoam Struktol J650; 60 g/L glycerol) were sterilized in 3 L bioreactor vessels (DR03F; DASGIP, Switzerland). After sterilization, 4.35 mL/L trace element solution (PTM1; 6.0 g/L CuSO₄·5H₂O; 0.08 g/L NaI; 3.0 g/L MnSO₄·H₂O; 0.2 g/L Na₂MoO₄·2H₂O; 0.02 g/L H₃BO₃; 0.5 g/L

CoCl₂; 20.0 g/L ZnCl₂; 65.0 g/L FeSO₄·7H₂O; 0.2 g/L biotin; 5 mL/L H₂SO₄) were added and pH was set to 5.0 with concentrated ammonia solution. The precultures were transferred aseptically to the respective vessel (the inoculation volume was 10%) and a batch phase on glycerol was carried out at 30°C with the stirrer fixed at 900 rpm. Aeration with compressed dry air was set to 1 vvm. Dissolved oxygen (dO₂) was measured with a sterilizable VisiFerm DO 225 probe (Hamilton, Switzerland) and controlled to be higher than 20%. The pH was measured with a sterilizable electrode (Mettler Toledo, Switzerland) and maintained constant at pH 5.0. Reactor weight was continuously recorded by a precision balance (Sartorius, Germany). Following complete glycerol consumption as indicated by an increase in the offgas signal, temperature was lowered to 25°C and an adaptation pulse of 0.5% (v/v) methanol (containing 4.35 mL/L PTM1) was added. After adaptation, 1.0% (v/v) methanol was pulsed repeatedly (for an example see Additional file 4). Before and after each pulse samples were taken and analyzed for OD₆₀₀, dry cell weight, HRP activity, extracellular protein content and methanol concentration.

Analysis of growth and expression parameters

Dry cell weight (DCW) was determined by centrifugation of 5 mL fermentation broth (4500 rpm, 10 min, 4°C), washing the pellet with 5 mL water and subsequent drying to a constant weight at 105°C. HRP activity in the cell-free supernatant was determined using a previously described assay [23] in a CuBiAn-XC enzymatic robot (OptoCell, Germany). Protein concentration was determined using a Bradford protein assay kit (Thermo Scientific, USA). All growth and protein expression parameters were determined in duplicates.

Analysis of substrate concentration

Methanol concentration in the cell-free supernatant was determined by HPLC as described previously [22]. Glycerol concentration was measured from cell-free samples in the CuBiAn-XC enzymatic robot. The device was calibrated with water-diluted glycerol standards ranging from 0 to 0.25 g/L. Samples with higher glycerol concentrations were automatically diluted by the system.

Calculation of strain physiological characteristics

Physiologically relevant parameters for characterization of the different yeast strains cultivated at different conditions and for quantification of the bioprocess were: Carbon dioxide evolution rate (CER; mmol/L/h), biomass yield ($Y_{X/MeOH}$; C-mol/C-mol), carbon dioxide yield ($Y_{CO_2/MeOH}$; C-mol/C-mol), C-balance, specific methanol uptake rate (q_{MeOH} ; mmol/g/h), specific productivity (q_p ; U/g/h), volumetric productivity (r_p ; U/L/h), specific activity (U/mg). All details on the calculation of these parameters have been published previously [34].

Additional files

Additional file 1: *Pichia pastoris* CBS 7435 HEM open reading frames. The single nucleotide polymorphism of *HEM15*, T918, is marked in grey.

Additional file 2: Co-overexpression of eGFP from PAOX1. The principal applicability of the employed co-overexpression construct is demonstrated indirectly by eGFP fluorescence of the strains co-overexpressing HRP and eGFP. The first two bars represent the starting strain, *PpMutS*, and the benchmark strain as controls. Fluorescence was determined at ex/em 488/507 nm, and normalized to the OD600 to account for potential growth differences.

Additional file 3: Co-overexpressions of HRP with either *HEM1* or *HEM3* from either PAOX1 or PCAT1. 1, co-overexpression of *HEM1* or *HEM3* from PAOX1; 2, co-overexpression of *HEM1* or *HEM3* from PCAT1. White bars, eGFP co-overexpression control; black bars, *HEM1* co-overexpression; gray bars, *HEM3* co-overexpression. The benchmark strain was included in all cultivations and set to 100% as reference (first bars).

Additional file 4: Batch cultivations of three recombinant *P. pastoris* strains. 1, benchmark strain; 2, *HEM1* strain; 3, *HEM3* strain; a/c/e, CER signal (solid line) and specific methanol uptake rate (open circles); b/d/f, carbon dioxide yields ($Y_{CO_2/S}$; grey squares) and biomass yields ($Y_{X/S}$; black circles).

Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; ALA: 5-aminolevulinic acid; AOX1: Alcohol oxidase 1 gene; CER: Carbon evolution rate; 96-DWP: 96-deep well plate; eGFP: Enhanced green fluorescent protein; HRP: Horseradish peroxidase; YNB: Difco™ yeast nitrogen base.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FWK, SC, TV and OS designed the experiments, analyzed and interpreted the data. FWK, SC, MJ, MGE and TV performed the experiments. FWK, SC and OS wrote the manuscript. FWK, AG, CH and OS conceived of the study. AG, CH and OS supervised the research. All authors read and approved the final manuscript.

Authors' information

FWK, TV, MGE, AG: Graz University of Technology, Institute of Molecular Biotechnology, NAWI Graz, Petersgasse 14, 8010 Graz, Austria. SC, MJ, CH, OS: Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, 1060 Vienna, Austria.

Acknowledgements

The authors would like to acknowledge support from NAWI Graz and the Austrian Science Fund FWF (projects: P24861-B19 and W901 DK Molecular Enzymology). This research was also funded by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement N°266025 (<http://bionexgen-fp7.eu>).

Author details

¹Graz University of Technology, NAWI Graz, Institute of Molecular Biotechnology, Graz, Austria. ²Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, 1060 Vienna, Austria.

Received: 29 October 2014 Accepted: 26 December 2014

Published online: 13 January 2015

References

- Franken ACW, Lokman BC, Ram AFJ, Punt PJ, van den Hondel CAMJJ, De Weert S. Heme biosynthesis and its regulation: towards understanding and improvement of heme biosynthesis in filamentous fungi. *Appl Microbiol Biotechnol*. 2011;91:447–60.
- Heinemann IU, Jahn M, Jahn D. The biochemistry of heme biosynthesis. *Arch Biochem Biophys*. 2008;474:238–51.
- Layer G, Reichelt J, Jahn D, Heinz DW. Structure and function of enzymes in heme biosynthesis. *Prot Sci*. 2010;19:1137–61.
- Franken ACW, Lokman BC, Ram AFJ, van den Hondel CAMJJ, de Weert S, Punt PJ. Analysis of the role of the *Aspergillus niger* aminolevulinic acid synthase (hemA) gene illustrates the difference between regulation of yeast and fungal haem- and sirohaem-dependent pathways. *Fems Microbiol Lett*. 2012;335:104–12.
- Franken ACW, Werner ER, Haas H, Lokman BC, van den Hondel CAMJJ, Ram AFJ, et al. The role of coproporphyrinogen III oxidase and ferrochelatase genes in heme biosynthesis and regulation in *Aspergillus niger*. *Appl Microbiol Biotechnol*. 2013;97:9773–85.
- Li FF, Wang Y, Gong K, Wang Q, Liang QF, Qi QS. Constitutive expression of RyhB regulates the heme biosynthesis pathway and increases the 5-aminolevulinic acid accumulation in *Escherichia coli*. *Fems Microbiol Lett*. 2014;350:209–15.
- Hoffman M, Gora M, Rytka J. Identification of rate-limiting steps in yeast heme biosynthesis. *Biochem Biophys Res Comm*. 2003;310:1247–53.
- Martinez JL, Liu L, Petranovic D, Nielsen J. Engineering the oxygen sensing regulation results in an enhanced recombinant human hemoglobin production by *Saccharomyces cerevisiae*. *Biotechnol Bioeng*. 2015;1:181–8.
- Michener JK, Nielsen J, Smolke CD. Identification and treatment of heme depletion attributed to overexpression of a lineage of evolved P450 monooxygenases. *Proc Natl Acad Sci U S A*. 2012;109:19504–9.
- Woodard SI, Dailey HA. Regulation of heme-biosynthesis in *Escherichia coli*. *Arch Biochem Biophys*. 1995;316:110–5.
- Liu LF, Martinez JL, Liu ZH, Petranovic D, Nielsen J. Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in *Saccharomyces cerevisiae*. *Metab Eng*. 2014;21:9–16.
- Krishnamurthy P, Xie T, Schuetz JD. The role of transporters in cellular heme and porphyrin homeostasis. *Pharmacol Ther*. 2007;114:345–58.
- Nishimoto M, Clark JE, Masters BSS. Cytochrome-P450-4a4 - expression in *Escherichia coli*, purification, and characterization of catalytic properties. *Biochem*. 1993;32:8863–70.
- Conesa A, van den Hondel CAMJJ, Punt PJ. Studies on the production of fungal peroxidases in *Aspergillus niger*. *Appl Environ Microbiol*. 2000;66:3016–23.
- Varnado CL, Goodwin DC. System for the expression of recombinant hemoproteins in *Escherichia coli*. *Prot Expr Purif*. 2004;35:76–83.
- Segura MD, Levin G, Miranda MV, Mendive FM, Targovnik HM, Cascone O. High-level expression and purification of recombinant horseradish peroxidase isozyme C in SF-9 insect cell culture. *Process Biochem*. 2005;40:795–800.
- Morawski B, Lin ZL, Cirino PC, Joo H, Bandara G, Arnold FH. Functional expression of horseradish peroxidase in *Saccharomyces cerevisiae* and *Pichia pastoris*. *Protein Eng*. 2000;13:377–84.
- Kueberl A, Schneider J, Thallinger GG, Anderl I, Wibberg D, Hajek T, et al. High-quality genome sequence of *Pichia pastoris* CBS7435. *J Biotechnol*. 2011;154:312–20.
- Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, et al. The COG database: an updated version includes eukaryotes. *BMC Bioinformatics*. 2003;4:41.
- Vogl T, Glieder A. Regulation of *Pichia pastoris* promoters and its consequences for protein production. *N Biotechnol*. 2013;30:385–404.
- Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T. Expression in the yeast *Pichia pastoris*. *Methods Enzymol*. 2009;463:169–89.
- Dietzsch C, Spadiut O, Herwig C. A fast approach to determine a fed batch feeding profile for recombinant *Pichia pastoris* strains. *Microb Cell Fact*. 2011;10:85.
- Dietzsch C, Spadiut O, Herwig C. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. *Microb Cell Fact*. 2011;10:14.
- Krainer FW, Dietzsch C, Hajek T, Herwig C, Spadiut O, Glieder A. Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. *Microb Cell Fact*. 2012;11:22.
- Capone S, Pletzenauer R, Maresch D, Metzger K, Altmann F, Herwig C, et al. Glyco-variant library of the versatile enzyme horseradish peroxidase. *Glycobiol*. 2014;24:852–63.
- Jiang H, Morgan JA. Optimization of an in vivo plant P450 monooxygenase system in *Saccharomyces cerevisiae*. *Biotechnol Bioeng*. 2004;85:130–7.
- Morawski B, Quan S, Arnold FH. Functional expression and stabilization of horseradish peroxidase by directed evolution in *Saccharomyces cerevisiae*. *Biotechnol Bioeng*. 2001;76:99–107.

28. Arrese M, Carvajal E, Robison S, Sambunaris A, Panek A, Mattoon J. Cloning of the delta-aminolevulinic acid synthase structural gene in yeast. *Curr Genet*. 1983;7:175–83.
29. Spadiut O, Dietzsch C, Herwig C. Determination of a dynamic feeding strategy for recombinant *Pichia pastoris* strains. *Methods Mol Biol*. 2014;1152:185–94.
30. Spadiut O, Zalai D, Dietzsch C, Herwig C. Quantitative comparison of dynamic physiological feeding profiles for recombinant protein production with *Pichia pastoris*. *Bioprocess Biosyst Eng*. 2013. doi:10.1007/s00449-013-1087-z.
31. Näätsaari L, Mistlberger B, Ruth C, Hajek T, Hartner FS, Glieder A. Deletion of the *Pichia pastoris* KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. *PLoS One*. 2012;7:e39720.
32. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–10.
33. Abad S, Kitz K, Hormann A, Schreiner U, Hartner FS, Glieder A. Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. *Biotechnol J*. 2010;5:413–20.
34. Weis R, Luiten R, Skranc W, Schwab H, Wubbolts M, Glieder A. Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *Fems Yeast Res*. 2004;5:179–89.

**Submit your next manuscript to BioMed Central
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

