Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Biotechnology

New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*

Thomas Vogl[1], Franz S Hartner[2] and Anton Glieder[3]

Biopharmaceuticals are an integral part of modern medicine and pharmacy. Both, the development and the biotechnological production of biopharmaceuticals are highly cost-intensive and require suitable expression systems. In this review we discuss established and emerging tools for reengineering the methylotrophic yeast *Pichia pastoris* for biopharmaceutical production. Recent advancements of this industrial expression system through synthetic biology include synthetic promoters to avoid methanol induction and to fine-tune protein production. New platform strains and molecular cloning tools as well as *in vivo* glycoengineering to produce humanized glycoforms have made *P. pastoris* an important host for biopharmaceutical production.

Addresses
[1] Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, Graz A-8010, Austria
[2] Sandoz GmbH, Biochemiestrasse 10, A-6250 Kundl, Austria
[3] Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, Graz A-8010, Austria

Corresponding author: Glieder, Anton (anton.glieder@acib.at, a.glieder@tugraz.at)

Current Opinion in Biotechnology 2013, **24**:xx–yy

This review comes from a themed issue on **Pharmaceutical biotechnology**

Edited by **Federico Gago** and **Ajikumar Parayil Kumaran**

0958-1669/$ – see front matter,

http://dx.doi.org/10.1016/j.copbio.2013.02.024

Introduction

Biopharmaceuticals are indispensable in modern medicine. The estimated market value is $70 to 80 billion (depending on the definition) and annual growth rates between 7 and 15% are expected [1–3]. This is another major reason for the worldwide focus of pharmacy and biotechnology on biopharmaceutical development and production. By definition, the term 'biopharmaceutical' refers to recombinant therapeutic proteins and nucleic acid based products and in the broader sense also to engineered cell or tissue-based products [2]. Vaccines, interferons and hormones like insulin, human growth hormone (hGH) and erythropoietin (EPO) are examples for protein biopharmaceuticals. Antibodies (including fragments like Fabs, scFvs and nanobodies) represent the biggest group of protein biopharmaceuticals [1–3].

Therapeutic proteins are typically produced in mammalian cell lines and *Escherichia coli*. While bacterial systems exhibit fast and robust growth in bioreactors using simple media, mammalian cells resemble their human counterparts more closely in terms of typical eukaryotic post translation modifications (PTMs) like glycosylation [2,4–6]. However, mammalian cell culture processes are relatively slow, require complex media, and are susceptible viral contaminations (Table 1).

Using yeasts enables to combine robust growth on simple media (in large scale bioreactors) with easily achievable genetic modifications and the introduction of the desired PTMs [7].

The 'classic' yeast *Saccharomyces cerevisiae* is one of the best studied eukaryotes and has been used as expression host for biopharmaceuticals since the early days of genetic engineering and recombinant protein production [8••]. Recently, the first biopharmaceutical produced in the methylotrophic yeast *Pichia pastoris* has been approved by the FDA (Kalbitor by Dyax Corp., a Kallikrein inhibitor) [1]. *P. pastoris* features all favorable traits of yeasts mentioned and has successfully been used to produce high titers of numerous heterologous proteins [7,9,10••]. Additionally, *P. pastoris* is suitable for high cell density cultivations, reaching more than 150 g dry cell weight per liter [11] and has high secretory capabilities for heterologous proteins, while secreting only low amounts of endogenous proteins (Table 1) [12].

In this review we focus on new opportunities for biopharmaceutical production by reengineered *P. pastoris* employing new tools, (semi-) synthetic parts and PTM pathways (see Figure 1). We also summarized already published approaches to identify regulatory elements and to reengineer promoters for bottom-up regulatory circuit design.

Recent developments in synthetic biology have extended the toolset of classical genetic engineering [13]. Tailor-made expression systems have been created by modifying transcription, translation, PTMs and designing synthetic regulatory networks [14,15••].

Glycoengineering

The majority of therapeutic proteins contain post-translational modifications, with glycosylation being the most common and at the same time the most complex PTM [2].

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Table 1

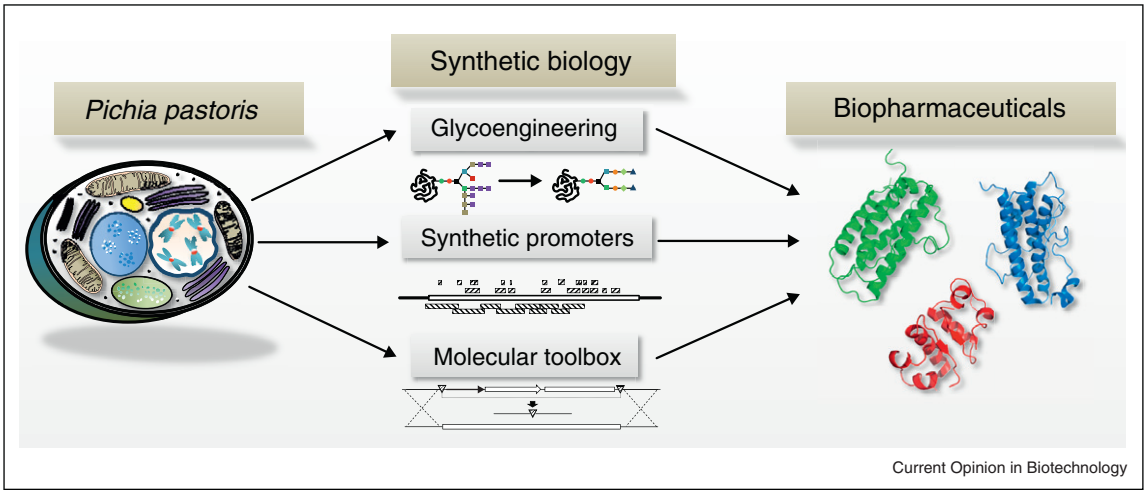
Comparison of expression systems used for biopharmaceutical production [4,6,7]

	Higher eukaryotes		Yeast		*Escherichia coli*
Ease of genetic modifications	Moderate		Simple		Simple
Cultivation	Slow growth rates, expensive complex (or synthetic) media required		Fast and robust growth, defined minimal media		Fastest growth, defined minimal media
Contaminations	Risk of viral contaminations, viral clearance required		Little risks of endotoxins or viral DNAs		Endotoxins presence requires thorough purification, possible phage infections
Post translational modifications (PTMs)	Closely resembling human PTMs; usually mixtures of several glycoform variants		Most human PTMs achievable, but natural glycosylation patterns differ from humans, hypermannosylation, engineered strains can achieve human glycoforms and high uniformity		Limited set of PTMs, some human PTMs (e.g. glycosylation) difficult to achieve
Protein yields and secretory capacities	High yields, highly efficient secretion, high specific productivity		High yields, secretory capacities depending on the species		High expression capacities, secretion mostly inefficient, extensive purification and downstream processing required
Most commonly used species	Mammalian cells	Insect cells	*Pichia pastoris*	*Saccharomyces cerevisiae*	
Recently approved biopharmaceuticals[a]	32	2	2[b]	4	17
Additional information and specific differences between host species of the same class	Commonly used cell lines: CHO (Chinese Hamster Ovary), BHK (baby hamster kidney), murine-myeloma-derived NS0, SP2/0 cell lines [2] and HEK293	Baculo virus based systems most commonly used for transfection Easy scale up Contaminations less problematic Mammalianized glycosylation [5]	Efficient and selective secretion, often higher protein titers than *S. cerevisiae*, for example, [8••] Crabtree negative, high cell density cultivations GRAS status Hypermannosylation is less pronounced in *P. pastoris* and critical terminal α-1,3-mannose linkages were not observed [19], engineered strains providing fully humanized glycosylation not available for *S. cerevisiae*	Important eukaryotic model organism, high molecular- and cell biological knowledge Crabtree positive, leading to ethanol production	Fastest efficient expression system Inexpensive Well established processes suitable for mass production Folding problems may lead to the formation of inclusion bodies and require expensive refolding (yet, inclusion bodies provide a valuable strategy to achieve high protein yields and simple purification) Inefficient acetate metabolism may hamper high cell density cultivation of some strains

[a] Data from Walsh [1], time period: January 2006–June 2010, in total 58 biopharmaceuticals have been approved, two biopharmaceuticals produced in transgenic animals were not listed.
[b] In this number Jetrea by ThromboGenics is included (approved in 2012 and not listed by Walsh [1]).

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Figure 1



Current synthetic biology approaches to improve biopharmaceutical yields and quality in *P. pastoris*. Glycoengineered strains provide humanized *N*-glycosylation patterns [14,15,16•], synthetic promoters allow the fine-tuning of expression levels [41,42,43•] and various tools for strain engineering [47–49,50•] and metabolic modeling [55•,56•,57•] are available.

Yeasts can perform typical eukaryotic PTMs, but final glycosylation patterns of yeasts and humans differ significantly. Hypermannosylation and terminal α-1,3-mannose linkages associated with glycoproteins from *S. cerevisiae*, can result in poor serum half-life or even immunogenic effects of therapeutic proteins [2,16•]. Thus, there have been efforts to humanize yeast glycosylation which has been accomplished in *P. pastoris* (see [16•–18•] for reviews). Also hypermannosylation is less pronounced in *P. pastoris* and terminal α-1,3-mannose linkages are not observed [19].

Here, we focus on recent developments of glycoengineering in *P. pastoris* and highlight the synthetic biology approaches and the heterologous and chimeric enzymes used for this purpose.

Achieving humanized glycosylation in yeast required on the one hand the elimination of hyperglycosylation by deleting the appropriate yeast genes, but on the other hand also the introduction of additional glycosidases and glycosyltransferases, including missing biosynthetic pathways and transporters for sugars not present in yeast, for example, sialic acid. In the case of galactose, UDP-glucose was converted to UDP-galactose in the Golgi by providing the respective epimerase activity [16•,17•].

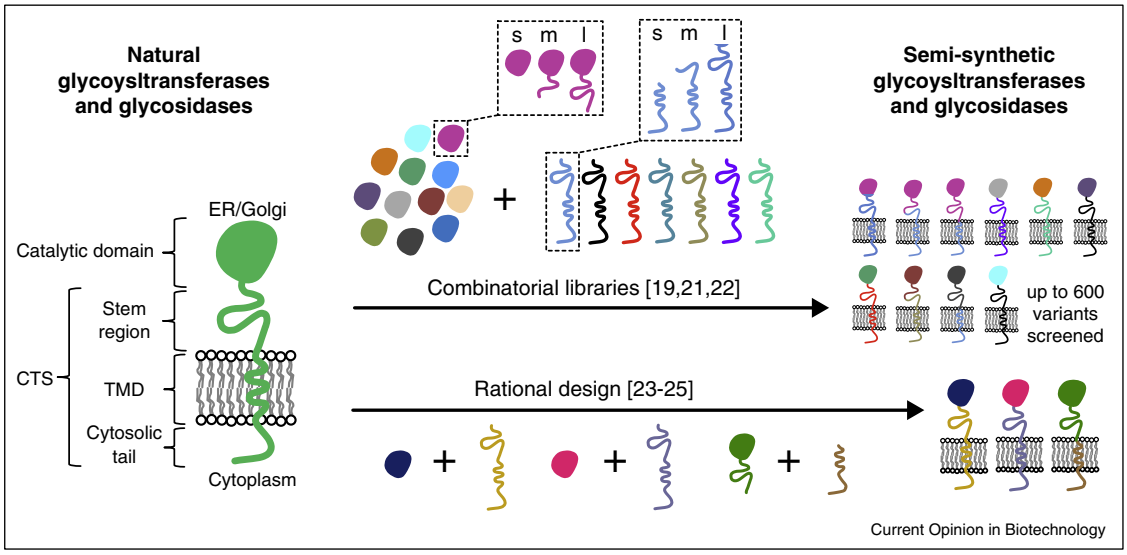
In addition to simple expression of these genes, correct spatial positioning along the secretory pathway in the ER and Golgi is essential, as the sequential activity of one enzyme produces the substrate for the next. To achieve the suitable positioning of the required factors along this cellular assembly line in *P. pastoris*, synthetic glycobiology [20] approaches were used.

Tailor-made glycosyltransferases and glycosidases with the desired catalytic properties and localization characteristics were created [19,21–25]. The strategy was based on the knowledge, that eukaryotic glycosyltransferases and glycosidases are type II membrane proteins, consisting of an N-terminal cytoplasmic tail, a membrane anchor domain, a stem region and a C-terminal catalytic domain (see Figure 2) [20]. The C-terminal catalytic domain is active independently of the localization conferring N-terminal part, which is also termed 'CTS' (cytoplasmic, transmembrane, stem). Fusions of catalytic domains to CTS fragments allowed the creation of semi-synthetic glycosyltransferases and glycosidases. A combinatorial library approach paired with a high-throughput screening was used to create and evaluate these proteins [19,21,22]. Rational design led to similar results [23–25], but eventually input from combinatorial libraries was also used [25].

Notably, the initial publications of the combinatorial libraries [19,21] contained barely any information on their composition and how the chimeric glycosyltransferases were designed. More recently, a comprehensive report about the catalytic domains, the CTS fragments, and how they were fused was published [26••]. The authors had not only started from a large set of 33 catalytic domains from different eukaryotes (e.g. fungi, worm, fruit fly, mouse, rat, human) and 66 fungal leader sequences, but also tested fusions of various lengths of both the catalytic domain and the CTS. Up to 600 variants were screened for optimal desired activity and localization along the generated artificial glycosylation pathways in *P. pastoris* (see Figure 2).

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Figure 2



Design strategies to create semi-synthetic glycosyltransferases and glycosidases for glycoengineering. On the left side, the general domain structure of glycosyltransferases and glycosidases is shown. These type II membrane proteins consist of an N-terminal cytosolic tail, a transmembrane domain (TMD), a stem region (these elements are referred to as CTS), and a C-terminal catalytic domain. In the middle and on the right side, design strategies for creating tailor-made enzymes with the desired catalytic activity and the proper localization in the sec pathway are shown. The combinatorial library approach involved the combination of large sets of catalytic domains with CTS fragments to fusion proteins, which were then screened for the desired activity [19,21,22]. Different lengths of the catalytic domains and the CTS fragments were tested (referred to as 's' for short, 'm' for medium, 'l' for long and shown exemplarily for one catalytic domain and one CTS). Rational approaches were also used to design these chimeric enzymes [23–25]. The schematic for the domain architecture and the combinatorial libraries is based on Czlapinski *et al*. [20] and Nett *et al*. [26••].

An essential milestone was achieved in 2006 by introduction of nine synthetic genes and deletion of six endogenous genes enabling the production of complex terminally sialylated glycoproteins in *P. pastoris* [22]. In the last five years, *N*-glycosylation site occupancy has been increased from 75–85% to 99% [27] and undesired β-linked mannose residues have been removed by creating a *P. pastoris* quadruple knock-out devoid of all four endogenous β-mannosyl transferases [28]. Furthermore, the production processes using glycoengineered *P. pastoris* strains have been optimized [29–31], antibody production in glycoengineered strains reached the g/l scale [32,33] and glycoengineered strains have also been established for surface display applications [34,35].

In addition to human like microbial glycosylation such heterologous synthetic pathways allow direct control of the intricate glycosylation process. Thereby, tailor-made glycoforms of a protein can be produced which can exhibit moderately differing pharmacodynamics. For example an antibody expressed in glycoengineered *P. pastoris* with a uniform, single glycoform showed improved antibody-mediated effector functions, compared to mammalian cell culture derived glycoforms with variable glycosylation patterns [36]. Therefore, better than nature glycoengineered *P. pastoris* strains pave the way for the creation of synthetic, supernatural glycoform preparations with altered properties compared to naturally occurring variants.

Synthetic promoters

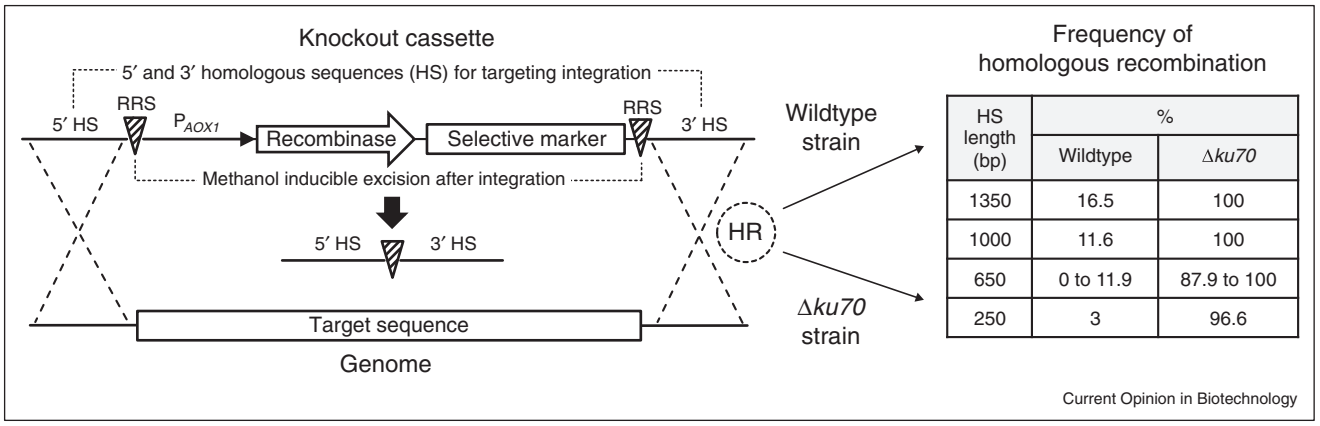
Efficient transcription is a critical step in gene expression. Therefore strong and controllable promoters are an essential tool for high titers in recombinant protein production [7,37]. In addition to natural promoters there has been a growing interest in synthetic promoters driving enhanced expression, improving folding or showing tailor-made regulatory profiles [37–39]. In *P. pastoris*, up to 22 g/l intracellular protein and 15 g/l secreted protein have been obtained with the most frequently applied, tightly controlled, strong and methanol inducible *AOX1* promoter (P_{AOX1}) [40].

As result, this promoter was the starting point for creating synthetic variants with increased promoter strength and altered, methanol free regulation, as the use of toxic and flammable methanol can cause a considerable safety risk in industrial processes.

One semi-rational approach to create synthetic P_{AOX1} variants relied on an *in silico* analysis for putative conserved eukaryotic transcription factor binding sites (TFBS) in P_{AOX1}. Subsequently, the respective short

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Figure 3



HS length (bp)	%	
	Wildtype	Δku70
1350	16.5	100
1000	11.6	100
650	0 to 11.9	87.9 to 100
250	3	96.6

Recombinase based self-excisable knockout cassettes for marker regeneration (left side). Increased rates of homologous recombination in a *P. pastoris* Δ*ku70* strain (right side). The knockout cassettes consist of a recombinase (Cre or FLP [48,49,50•]) and a marker gene flanked by the respective recombinase recognition sites and are directed to the genome via the 5′ and 3′ homologous sequences to delete the desired target sequence. After integration via a double cross-over event, self-excision of the recombinase and the marker gene can be initiated by the expression of the recombinase from the methanol inducible *AOX1* promoter (P_{AOX1}), leaving only the recombinase recognition site in the genome (notably Marx *et al*. [49] provided the recombinase transiently on a CEN/ARS plasmid). The initial integration in the genome is dependent on homologous recombination (HR). Exemplary frequencies of homologous recombination (in %) of the wildtype compared to the Δ*ku70* strain are shown (right side). The length of the homologous sequence indicates the number of base pairs (bp) added on both sides of the cassette [50•]. For 650 bp two different integration loci were tested, therefore two % values are given.

sequence stretches were deleted [41]. These deletion variants showed both increased and decreased reporter gene expression levels spanning 6–160% of wildtype P_{AOX1} driven expression. Alternative approaches relied on the systematic deletion of larger adjacent fragments of almost the entire promoter [42]. Surprisingly, some small deletions and point mutations resulted in altered regulation as these variants were moderately active when glucose was depleted, without requiring the inductor methanol [41]. This derepression effect was further optimized by combinations of deletions and insertions of important sequence stretches. Such altered induction properties now enable the consecutive induction of coexpressed proteins such as chaperons and the therapeutic protein of interest. Putative TFBS of P_{AOX1} were also fused to natural core promoter fragments to create short semi-synthetic variants, which again showed altered regulation and surpassed the full-length wildtype promoter in certain applications especially when multiple copies of the expression cassettes were integrated [41,43•]. Also the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (P_{GAP}) of *P. pastoris* has been engineered by a random mutagenesis approach [44] showing the potential of additional promoters for expression fine tuning or the generation of new regulatory circuits. Bio-process strategies for biotechnologically relevant enzymes have been improved by employing these synthetic promoters [41,43•,44,45] and similar effects can be expected for biopharmaceuticals. Furthermore, multiple positive and negative factors involved in P_{AOX1} regulation have been identified since this initial semi-rational promoter engineering (see [40] for a recent review), opening the way for the design of novel synthetic regulatory circuits for gene expression and pathway design.

Molecular toolbox for synthetic biology in *P. pastoris*

Synthetic biology applications require efficient tools for strain engineering. For example, the creation of *P. pastoris* strains providing a fully humanized glycosylation pattern necessitated in the first place the development of suitable genetic strategies to knock out and introduce multiple genes [46]. Efficient strategies for gene replacements and marker recycling have now become available for *P. pastoris*. Namely, systems based on new counter selective markers [47], a Cre/loxP strategy [48,49] and an advanced flipper cassette application [50•] have been reported and applied. The recombinase based strategies [48,49,50•] allow active excision of the marker gene used in a deletion cassette and to thereby recycle markers and perform sequential rounds of deletions. This is achieved by designing a deletion cassette, in which the marker gene and the recombinase are flanked by two recombinase recognition sites and the recombinase is placed under the tight control of the methanol inducible *AOX1* promoter (see Figure 3). Näätsaari *et al*. [50•] applied such a strategy to generate a new platform of *P. pastoris* expression strains and Marx *et al*. [49] boosted riboflavin production in *P. pastoris* by subsequently overexpressing all six genes of the riboflavin biosynthetic pathway by inserting the strong constitutive *GAP* promoter upstream of

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

these genes. Coupling such approaches with synthetic promoter variants [41,42,43•,44] might support the transcriptional fine tuning of individual enzyme activities of biosynthetic pathways.

Site specific integration and knock-out strain generation rely on endogenous homologous recombination. While in *S. cerevisiae* HR is working highly efficiently, non-homologous end joining is the preferred pathway in most other filamentous fungi and yeasts, including *P. pastoris*. HR occurs at less than 1% and up to 30% of all integration events, depending on the length of the homologous targeting sequence [50•]. For example during glycoengineering of *P. pastoris* only 5 out of 460 clones showed the desired gene replacement [46]. Targeted integration and deletion should become more efficient in the future by employing a *P. pastoris ku70* deletion strain with increased rates of HR [50•]. By the deletion of a Ku70 homologue, a protein involved in NHEJ, HR rates of up to 100% were achieved (see Figure 3). The Δ*ku70* strain did not show genetic instability, but the growth rates were 10–30% lower than those of the wildtype (depending on the carbon source) and the strain showed a decreased survival rate under UV light. This hints an increased susceptibility to DNA damage and complementing the wildtype *KU70* gene after completion of strain engineering was recommended [50•].

In addition to precise deletions, site specific integration, and marker recycling, new cloning techniques facilitate the construction of the respective gene expression and deletion constructs. Efficient *in vitro* recombination methods such as Gibson assembly [51] enable flexible restriction free cloning and library generation allowing the simple testing of libraries of promoters, artificial or natural expression enhancers and signal-sequences or other targeting sequences. Although, bottom up approaches to design individual parts for *P. pastoris* strain reengineering and expression cassette constructions are ongoing, there is no systematic synthetic biology parts collection for this yeast so far.

Bioinformatics tools complete the toolbox for synthetic biology applied in *P. pastoris*. High-quality genome sequences [52–54] and metabolic models [55•,56•,57•] of *P. pastoris* have recently become available. This comprehensive new background knowledge enables research towards systems wide understanding of the *P. pastoris* expression system and provides the basis for reengineering this host using synthetic parts and pathways to improve biopharmaceutical production. For example, recent studies in *P. pastoris* have hinted an interconnection of both the carbon metabolism [58] and the cellular redox state [59] with protein production and secretion. Thus, similar to *S. cerevisiae* [60,61], a systems biology view on secretion coupled with a synergistic use of metabolic engineering and synthetic biology approaches [62,63] promise coming improvements for biopharmaceutical production by *P. pastoris*.

Conclusions

Over the last two decades, *P. pastoris* has been established as one of the most frequently used expression systems in both industry and academia. Beside a large number of various enzymes, many human proteins and biopharmaceuticals were also efficiently produced by *P. pastoris*. The adaptation of the yeast high-mannose type glycosylation to the complex humanized glycosylation was a major achievement and resulted in uniform glycoforms from microbial production. Synthetic promoter variants with altered regulatory profiles and expression levels surpassed their natural counterparts for enzyme production. Equally, these variants can be used to optimize and fine-tune the expression of therapeutic proteins. Also other new key methodologies for synthetic biology such as efficient gene deletion and assembly strategies, metabolic models and strains with altered recombination properties have become available. Together with milestones such as the FDA approval, these new tools and techniques have a high potential to boost the production of biopharmaceuticals and for efficient metabolic engineering (see Box 1).

Altered and new biosynthetic pathways for posttranslational modifications such as precise glycosylation are enabling techniques giving access to new therapeutics with uniform and excellent quality.

Synthetic biology will certainly not only further improve industrial enzyme production, but also stimulate and facilitate innovative approaches for biopharmaceutical production in *P. pastoris*.

Box 1 Milestones and recent accomplishments for biopharmaceutical production in *P. pastoris*

(1) FDA GRAS (generally regarded as safe) status in 2006 (Phospholipase C by Diversa Corp., for degumming vegetables oils for food use).

(2) FDA approved biopharmaceutical production processes in 2009 (Kalbitor by Dyax Corp., a Kallikrein inhibitor) and 2012 (Jetrea by ThromboGenics NV, for the treatment of vitreomacular traction).

(3) Glycoengineered strains providing humanized, uniform *N*-glycosylation patterns [22,25].

(4) Synthetic promoters for fine-tuning expression levels [41,42,43•].

(5) Efficient strategies for knockouts of multiple genes and overexpression of entire pathways [48,49,50•].

(6) High quality genome sequences [52–54].

(7) Establishment of *in silico* metabolic models for strain engineering [55•,56•,57•].

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Acknowledgements

T.V. is financed by theEuropean Union's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 289646 (Kyrobio). We also gratefully acknowledge the Austrian Science Fund (FWF) project number W901 (DK 'Molecular Enzymology' Graz) and the Austrian Centre of Industrial Biotechnology (ACIB) contribution was supported by FFG, bmvit, mvwfi, ZIT, Zukunftsstiftung Tirol and Land Steiermark within the Austrian COMET programme (FFG grant 824186). We would also like to thank Andrea Camattari and Claudia Ruth for valuable discussions.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Walsh G: **Biopharmaceutical benchmarks 2010**. *Nat Biotechnol* 2010, **28**:917-924.

2. Walsh G: **Post-translational modifications of protein biopharmaceuticals**. *Drug Discov Today* 2010, **15**:773-780.

3. Goodman M: **Market watch: sales of biologics to show robust growth through to 2013**. *Nat Rev Drug Discov* 2009, **8**:837.

4. Demain AL, Vaishnav P: **Production of recombinant proteins by microbes and higher organisms**. *Biotechnol Adv* 2009, **27**:297-306.

5. Palmberger D, Klausberger M, Berger I, Grabherr R: **MultiBac turns sweet**. *Bioengineered* 2012, **4**:1-6.

6. Berlec A, Strukelj B: **Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells**. *J Ind Microbiol Biotechnol* 2013 http://dx.doi.org/10.1007/s10295-013-1235-0.

7. Mattanovich D, Branduardi P, Dato L, Gasser B, Sauer M, Porro D: **Recombinant protein production in yeasts**. *Methods Mol Biol* 2012, **824**:329-358.

8. •• Martínez JL, Liu L, Petranovic D, Nielsen J: **Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation**. *Curr Opin Biotechnol* 2012, **23**:965-971.
Concise overview of biopharmaceutical production in *S. cerevisiae*. Covers approaches for strain engineering and also shortly biopharmaceutical production in *P. pastoris*.

9. Daly R, Hearn MTW: **Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production**. *J Mol Recognit* 2005, **18**:119-138.

10. •• Gasser B, Prielhofer R, Marx H, Maurer M, Nocon J, Steiger M, Puxbaum V, Sauer M, Mattanovich D: ***Pichia pastoris*: protein production host and model organism for biomedical research**. *Future Microbiol* 2013, **8**:191-208.
Detailed overview on protein production in *P. pastoris* covering key aspects for high level expression, strain engineering, secretion and novel methods. Protocols are also provided and the use of *P. pastoris* as a model for human cell biology is summarized.

11. Jahic M, Veide A, Charoenrat T, Teeri T, Enfors S-O: **Process technology for production and recovery of heterologous proteins with *Pichia pastoris***. *Biotechnol Prog* 2006, **22**:1465-1473.

12. Damasceno LM, Huang C-J, Batt CA: **Protein secretion in *Pichia pastoris* and advances in protein production**. *Appl Microbiol Biotechnol* 2012, **93**:31-39.

13. Liang J, Luo Y, Zhao H: **Synthetic biology: putting synthesis into biology**. *Wiley Interdiscip Rev Syst Biol Med* 2012, **3**:7-20.

14. Lynch SA, Gill RT: **Synthetic biology: new strategies for directing design**. *Metab Eng* 2012, **14**:205-211.

15. •• Krivoruchko A, Siewers V, Nielsen J: **Opportunities for yeast metabolic engineering: lessons from synthetic biology**. *Biotechnol J* 2011, **6**:262-276.
Excellent, comprehensive review highlighting the use of synthetic biology to engineer *S. cerevisiae*. Describes approaches ranging from synthetic promoters, pathway engineering, riboswitches, protein engineering to synthetic regulatory networks.

16. • De Pourcq K, De Schutter K, Callewaert N: **Engineering of glycosylation in yeast and other fungi: current state and perspectives**. *Appl Microbiol Biotechnol* 2010, **87**:1617-1631.
See annotation to Ref. [18•].

17. • Hamilton SR, Gerngross TU: **Glycosylation engineering in yeast: the advent of fully humanized yeast**. *Curr Opin Biotechnol* 2007, **18**:387-392.
See annotation to Ref. [18•].

18. • Bollok M, Resina D, Valero F, Ferrer P: **Recent patents on the *Pichia pastoris* expression system: expanding the toolbox for recombinant protein production**. *Recent Pat Biotechnol* 2009, **3**:192-201.
Refs. [16•,17•,18•] provide an in-depth coverage of the glycoengineering efforts in *P. pastoris* before the period under review. The most recent reviews from the two main groups involved (of Gerngross [17•] and Callewaert [16•]) and a review covering patent issues [18•] are listed.

19. Choi B-K, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, Miele RG, Nett JH, Wildt S, Gerngross TU: **Use of combinatorial genetic libraries to humanize *N*-linked glycosylation in the yeast *Pichia pastoris***. *Proc Natl Acad Sci U S A* 2003, **100**:5022-5027.

20. Czlapinski JL, Bertozzi CR: **Synthetic glycobiology: exploits in the Golgi compartment**. *Curr Opin Chem Biol* 2006, **10**:645-651.

21. Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H *et al.*: **Production of complex human glycoproteins in yeast**. *Science* 2003, **301**:1244-1246.

22. Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi B-K *et al.*: **Humanization of yeast to produce complex terminally sialylated glycoproteins**. *Science* 2006, **313**:1441-1443.

23. Callewaert N, Laroy W, Cadirgi H, Geysens S, Saelens X, Min Jou W, Contreras R: **Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2-alpha-D-mannosidase for *N*-glycan engineering in *Pichia pastoris***. *FEBS Lett* 2001, **503**:173-178.

24. Vervecken W, Kaigorodov V, Callewaert N, Geysens S, De Vusser K, Contreras R: **In vivo synthesis of mammalian-like, hybrid-type *N*-glycans in *Pichia pastoris***. *Appl Environ Microbiol* 2004, **70**:2639-2646.

25. Jacobs PP, Geysens S, Vervecken W, Contreras R, Callewaert N: **Engineering complex-type *N*-glycosylation in *Pichia pastoris* using GlycoSwitch technology**. *Nat Protoc* 2009, **4**:58-70.

26. •• Nett JH, Stadheim TA, Li H, Bobrowicz P, Hamilton SR, Davidson RC, Choi B, Mitchell T, Bobrowicz B, Rittenhour A *et al.*: **A combinatorial genetic library approach to target heterologous glycosylation enzymes to the endoplasmic reticulum or the Golgi apparatus of *Pichia pastoris***. *Yeast* 2011, **28**:237-252.
Revelation of the exact composition and design of the combinatorial genetic libraries (initially described in [19,21]) for the generation of the synthetic glycosyltransferases and glycosidases used to humanize *P. pastoris N*-glycosylation.

27. Choi B-K, Warburton S, Lin H, Patel R, Boldogh I, Meehl M, D'Anjou M, Pon L, Stadheim TA, Sethuraman N: **Improvement of *N*-glycan site occupancy of therapeutic glycoproteins produced in *Pichia pastoris***. *Appl Microbiol Biotechnol* 2012, **95**:671-682.

28. Hopkins D, Gomathinayagam S, Rittenhour AM, Du M, Hoyt E, Karaveg K, Mitchell T, Nett JH, Sharkey NJ, Stadheim TA *et al.*: **Elimination of β-mannose glycan structures in *Pichia pastoris***. *Glycobiology* 2011, **21**:1616-1626.

29. Jacobs PP, Inan M, Festjens N, Haustraete J, Van Hecke A, Contreras R, Meagher MM, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered *Pichia pastoris* under controlled specific growth rate**. *Microb Cell Fact* 2010, **9**:93.

30. Nett JH, Gomathinayagam S, Hamilton SR, Gong B, Davidson RC, Du M, Hopkins D, Mitchell T, Mallem MR, Nylen A *et al.*:

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024

Optimization of erythropoietin production with controlled glycosylation-PEGylated erythropoietin produced in glycoengineered *Pichia pastoris*. *J Biotechnol* 2012, **157**:198-206.

31. Berdichevsky M, D'Anjou M, Mallem MR, Shaikh SS, Potgieter TI: **Improved production of monoclonal antibodies through oxygen-limited cultivation of glycoengineered yeast**. *J Biotechnol* 2011, **155**:217-224.

32. Potgieter TI, Cukan M, Drummond JE, Houston-Cummings NR, Jiang Y, Li F, Lynaugh H, Mallem M, McKelvey TW, Mitchell T *et al.*: **Production of monoclonal antibodies by glycoengineered *Pichia pastoris***. *J Biotechnol* 2009, **139**:318-325.

33. Ye J, Ly J, Watts K, Hsu A, Walker A, McLaughlin K, Berdichevsky M, Prinz B, Sean Kersey D, D'Anjou M *et al.*: **Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production**. *Biotechnol Prog* 2011, **27**:1744-1750.

34. Jacobs PP, Ryckaert S, Geysens S, De Vusser K, Callewaert N, Contreras R: **Pichia surface display: display of proteins on the surface of glycoengineered *Pichia pastoris* strains**. *Biotechnol Lett* 2008, **30**:2173-2181.

35. Lin S, Houston-Cummings NR, Prinz B, Moore R, Bobrowicz B, Davidson RC, Wildt S, Stadheim TA, Zha D: **A novel fragment of antigen binding (Fab) surface display platform using glycoengineered *Pichia pastoris***. *J Immunol Methods* 2012, **375**:159-165.

36. Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Choi B-K, Cook WJ, Cukan M *et al.*: **Optimization of humanized IgGs in glycoengineered *Pichia pastoris***. *Nat Biotechnol* 2006, **24**:210-215.

37. Juven-Gershon T, Cheng S, Kadonaga JT: **Rational design of a super core promoter that enhances gene expression**. *Nat Methods* 2006, **3**:917-922.

38. Ruth C, Glieder A: **Perspectives on synthetic promoters for biocatalysis and biotransformation**. *Chembiochem* 2010, **11**:761-765.

39. Blount BA, Weenink T, Vasylechko S, Ellis T: **Rational diversification of a promoter providing fine-tuned expression and orthogonal regulation for synthetic biology**. *PLoS ONE* 2012, **7**:e33279.

40. Vogl T, Glieder A: **Regulation of *Pichia pastoris* promoters and its consequences for protein production**. *New Biotechnol* 2012 http://dx.doi.org/10.1016/j.nbt.2012.11.010.

41. Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A: **Promoter library designed for fine-tuned gene expression in *Pichia pastoris***. *Nucleic Acids Res* 2008, **36**:e76.

42. Xuan Y, Zhou X, Zhang W, Zhang X, Song Z, Zhang Y: **An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris***. *FEMS Yeast Res* 2009, **9**:1271-1282.

43. • Ruth C, Zuellig T, Mellitzer A, Weis R, Looser V, Kovar K, Glieder A: **Variable production windows for porcine trypsinogen employing synthetic inducible promoter variants in *Pichia pastoris***. *Syst Synth Biol* 2010, **4**:181-191.
Describes the use of P_{AOX1} deletion variants and the design of small synthetic promoters (by the fusion of *cis*-acting elements to core promoter fragments) to improve protein production.

44. Qin X, Qian J, Yao G, Zhuang Y, Zhang S, Chu J: **GAP promoter library for fine-tuning of gene expression in *Pichia pastoris***. *Appl Environ Microbiol* 2011, **77**:3600-3608.

45. Mellitzer A, Weis R, Glieder A, Flicker K: **Expression of lignocellulolytic enzymes in *Pichia pastoris***. *Microb Cell Fact* 2012, **11**:61.

46. Nett JH, Gerngross TU: **Cloning and disruption of the PpURA5 gene and construction of a set of integration vectors for the stable genetic modification of *Pichia pastoris***. *Yeast* 2003, **20**:1279-1290.

47. Yang J, Jiang W, Yang S: **mazF as a counter-selectable marker for unmarked genetic modification of *Pichia pastoris***. *FEMS Yeast Res* 2009, **9**:600-609.

48. Pan R, Zhang J, Shen W-L, Tao Z-Q, Li S-P, Yan X: **Sequential deletion of *Pichia pastoris* genes by a self-excisable cassette**. *FEMS Yeast Res* 2011, **11**:292-298.

49. Marx H, Mattanovich D, Sauer M: **Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris***. *Microb Cell Fact* 2008, **7**:23.

50. • 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.
Report on a *P. pastoris* Δ*ku70* strain with increased rates of homologous recombination, achieved by deleting a gene (*KU70*) coding for a protein involved in non-homologous end joining. Thereby access to deletions, which were otherwise difficult to obtain for *P. pastoris*, was facilitated. Contains also the description of self-excising knock out cassettes (similar to [47–49]) enabling subsequent rounds of strain engineering with only one selective marker.

51. Gibson DG, Young L, Chuang R, Venter JC, Hutchison CA, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases**. *Nat Methods* 2009, **6**:343-345.

52. De Schutter K, Lin Y-C, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouzé P, Van de Peer Y, Callewaert N: **Genome sequence of the recombinant protein production host *Pichia pastoris***. *Nat Biotechnol* 2009, **27**:561-566.

53. Mattanovich D, Graf A, Stadlmann J, Dragosits M, Redl A, Maurer M, Kleinheinz M, Sauer M, Altmann F, Gasser B: **Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris***. *Microb Cell Fact* 2009, **8**:29.

54. Küberl A, Schneider J, Thallinger GG, Anderl I, Wibberg D, Hajek T, Jaenicke S, Brinkrolf K, Goesmann A, Szczepanowski R *et al.*: **High-quality genome sequence of *Pichia pastoris* CBS7435**. *J Biotechnol* 2011, **154**:312-320.

55. • Chung BK, Selvarasu S, Andrea C, Ryu J, Lee H, Ahn J, Lee H, Lee D: **Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast *Pichia pastoris* for strain improvement**. *Microb Cell Fact* 2010, **9**:50.
See annotation to Ref. [57•].

56. • Sohn SB, Graf AB, Kim TY, Gasser B, Maurer M, Ferrer P, Mattanovich D, Lee SY: **Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production**. *Biotechnol J* 2010, **5**:705-715.
See annotation to Ref. [57•].

57. • Caspeta L, Shoaie S, Agren R, Nookaew I, Nielsen J: **Genome-scale metabolic reconstructions of *Pichia stipitis* and *Pichia pastoris* and in silico evaluation of their potentials**. *BMC Syst Biol* 2012, **6**:24.
Refs. [55•,56•,57•] describe metabolic models for two different *P. pastoris* strains GS115 [55•,57•] and DSMZ 70382 [56•] that can be used for *in silico* predictions as a basis for engineering approaches.

58. Heyland J, Fu J, Blank LM, Schmid A: **Carbon metabolism limits recombinant protein production in *Pichia pastoris***. *Biotechnol Bioeng* 2011, **108**:1942-1953.

59. Delic M, Rebnegger C, Wanka F, Puxbaum V, Haberhauer-Troyer C, Hann S, Köllensperger G, Mattanovich D, Gasser B: **Oxidative protein folding and unfolded protein response elicit differing redox regulation in endoplasmic reticulum and cytosol of yeast**. *Free Radic Biol Med* 2012, **52**:2000-2012.

60. Hou J, Tyo K, Liu Z, Petranovic D, Nielsen J: **Engineering of vesicle trafficking improves heterologous protein secretion in *Saccharomyces cerevisiae***. *Metab Eng* 2012, **14**:120-127.

61. Hou J, Tyo KEJ, Liu Z, Petranovic D, Nielsen J: **Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae***. *FEMS Yeast Res* 2012, **12**:491-510.

62. Nielsen J, Keasling JD: **Synergies between synthetic biology and metabolic engineering**. *Nat Biotechnol* 2011, **29**:693-695.

63. Yadav VG, De Mey M, Lim CG, Ajikumar PK, Stephanopoulos G: **The future of metabolic engineering and synthetic biology: towards a systematic practice**. *Metab Eng* 2012, **14**:233-241.

Please cite this article in press as: Vogl T, et al.: New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*, Curr Opin Biotechnol (2013), http://dx.doi.org/10.1016/j.copbio.2013.02.024