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Towards systems metabolic engineering in *Pichia pastoris*

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* is firmly established as a host for the production of recombinant proteins, frequently outperforming other heterologous hosts. Already, a sizeable amount of systems biology knowledge has been acquired for this non-conventional yeast. By applying various omics-technologies, productivity features have been thoroughly analyzed and optimized via genetic engineering. However, challenging clonal variability, limited vector repertoire and insufficient genome annotation have hampered further developments. Yet, in the last few years a reinvigorated effort to establish *P. pastoris* as a host for both protein and metabolite production is visible. A variety of compounds from terpenoids to polyketides have been synthesized, often exceeding the productivity of other microbial systems. The clonal variability was systematically investigated and strategies formulated to circumvent untargeted events, thereby streamlining the screening procedure. Promoters with novel regulatory properties were discovered or engineered from existing ones. The genetic tractability was increased via the transfer of popular manipulation and assembly techniques, as well as the creation of new ones. A second generation of sequencing projects culminated in the creation of the second best functionally annotated yeast genome. In combination with landmark physiological insights and increased output of omics-data, a good basis for the creation of refined genome-scale metabolic models was created. The first application of model-based metabolic engineering in *P. pastoris* showcased the potential of this approach. Recent efforts to establish yeast peroxisomes for compartmentalized metabolite synthesis appear to fit ideally with the well-studied high capacity peroxisomal machinery of *P. pastoris*. Here, these recent developments are collected and reviewed with the aim of supporting the establishment of systems metabolic engineering in *P. pastoris*.

1. Introduction

In principle, systems metabolic engineering and systems biotechnology aim to bring the predictability and model based approach of the engineering world to biological systems. For the application of systems metabolic engineering an organism thoroughly studied via omics-technologies, the availability of computational systems biology tools and the capability for targeted genetic engineering, including synthetic biology, is required (Keasling, 2010; Lee et al., 2012; S.Y. Lee et al., 2005; Lee and Kim, 2015). So far, the biotechnological bacterial workhorse *Escherichia coli*, the gram-positive model bacterium *Bacillus subtilis* and the model yeast *Saccharomyces cerevisiae* are the only microorganisms for which systems metabolic engineering is firmly established (Kelwick et al., 2014). Many other organisms show promising features, but not all requirements are fulfilled yet or systems metabolic engineering is currently being established. The very recent progress in *Pichia pastoris* research points towards a growing interest and effort to enable this approach in this yeast. By giving a brief overview of *P.*

Abbreviations: ADH, alcohol dehydrogenase; AOX1/2, alcohol oxidase 1/2; ARS, autonomously replicating sequence; ATF, artificial transcription factor; CDW, cell dry weight; ChiP, chromatin immunoprecipitation; COBRA, constraint-based reconstruction and analysis; CPR, cytochrome P450 reductase; CYP, cytochrome P450 monooxygenase; DAS1/2, dihydroxyacetone synthase isoform 1/2; DDS, dammarenediol-II synthase; DMAPP, dimethylallyl pyrophosphate; DSB, double-strand breaks; GAP, glyceraldehyde-3-phosphate; GCN, gene copy number; GEM, genome-scale metabolic model; GlcNAc, *N*-acetylglucosamine; gRNA, guide RNA; HR, homologous recombination; IPP, isopentenyl pyrophosphate; IR, inverted repeat; MIG1/2, multicopy inhibitor of GAL gene expression 1/2; MIT1, methanol-induced transcription factor 1; MUT, methanol utilization; MVA, mevalonate; MXR1, methanol expression regulator 1; NGS, next generation sequencing; NHEJ, non-homologous end joining; NRG1, negative regulator of glucose-repressed genes 1; OCH1, α-1,6-mannosyltransferase; ORF, open reading frame; PRM1, positive regulator of methanol 1; PTS, peroxisomal targeting sequence; RNA-Seq, RNA-sequencing; ROS, reactive oxygen species; S1,7BP, sedoheptulose-1,7-bisphosphate; UPR, unfolded protein response; VPS, vacuolar protein sorting; WSC, cell Wall integrity and Stress response Component; XYL5P, Xylulose-5-phosphate; ZF, zinc finger

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pastoris history, summarizing the newest findings in detail and describing potential future applications, this review intends to aid this line of research.

The methylotrophic, non-conventional budding yeast *P. pastoris* has been established as a wide-spread recombinant protein expression platform in both academia and the industry. According to the web platform www.pichia.com, over 5000 different proteins have been produced in this yeast. The popularity stems from the availability of simple and robust high-cell density cultivation procedures, tightly regulated and extraordinarily strong promoters, good post-translational modification and secretion capabilities, as well as ease of genetic manipulation (Ahmad et al., 2014; Macauley-Patrick et al., 2005). While early ventures focused on technical enzymes (Cereghino and Cregg, 2000), the acquisition of the FDA GRAS (generally regarded as safe) status (Ciofalo et al., 2006) promoted the development of biopharmaceuticals, e.g. the kallikrein inhibitor Kalbitor® or the aglycosylated protease Jetrea® (Corchero et al., 2013; Meehl and Stadheim, 2014). *P. pastoris* has demonstrated its suitability for the expression of targets that proved problematic in other host systems, e.g. membrane bound proteins (Byrne, 2015; Vogl et al., 2014) or glycoproteins (Laukens et al., 2015). Therefore, it has been recommended to consider *P. pastoris* as a standard tool for labs interested in the production of recombinant proteins (Bill, 2014).

Typically, the expression cassette harboring the target gene is integrated into a chromosomal locus via homologous recombination (HR), ensuring a high genetic stability (Cereghino and Cregg, 2000). Alternatively, episomal vectors using the native autonomously replicating sequences (ARS) *PARS1* and *PARS2* are available, but are merely used in a few applications (Cregg et al., 1985; C.C. Lee et al., 2005). While most protein expression studies only require the knock-in of a single target gene, the secretion of fully humanized and terminally sialylated glycoproteins was the largest genetic engineering project in *P. pastoris* to date (Hamilton et al., 2006). This project required deletion of four genes and the integration of 14 foreign genes, including the transfer of the complete human CMP-*N*-actelyneuraminic acid biosynthesis pathway. Other genetic engineering ventures include the introduction of biotin-prototrophy (Gasser et al., 2010), modification of the methanol utilization (MUT) pathway (Krainer et al., 2012) and improvement of protein folding and secretion features (Guerfal et al., 2010). Compared to the wealth of publications focused on protein production and its optimization, relatively few studies concern the biosynthesis of metabolites. The use of non-conventional yeasts in metabolic engineering projects in order to further the yeast platform as a whole was postulated. Only one example from *P. pastoris* was cited (Liu et al., 2013). Nevertheless, chemically different metabolites from riboflavin (Marx et al., 2008) and poly-3-hydroxybutyrate (Poirier et al., 2002) to different carotenoids (Araya-Garay et al., 2012a, 2012b) have been successfully produced in the past.

Although *P. pastoris* shares many properties with the conventional yeast *S. cerevisiae*, it also has its own distinguishing features that provide opportunities or present challenges. Genome integrations are stable but often a high variability of clones from one transformation is encountered, displaying various productivity characteristics or changes in their physiology (Cregg et al., 1985; Schwarzhans et al., 2016a, 2016b). A time-consuming screening process has to be employed in order to find the clone with the optimal features for the desired application (Looser et al., 2015). One of the causes for the clonal variability is the non-homologous end joining (NHEJ) pathway, which mediates integration of foreign DNA at untargeted locations. The clonal variability is named as a key factor holding back the further development of *P. pastoris* as a platform for producing value-added chemicals (Kelwick et al., 2014). On the other hand, *P. pastoris* inherently good protein production features, along with a Crabtree-negative phenotype and a well-developed and well-studied peroxisomal machinery, should make it a desirable host for metabolic engineering projects, surpassing *S. cerevisiae* in certain applications.

The methanol inducible alcohol oxidase 1 (*AOX1*) promoter *pAOX1* and the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter *pGAP* are the most popular choices for facilitating foreign gene expression (Vogl and Glieder, 2013). *pAOX1* offers tight regulation with near-zero transcriptional activity under repressed or derepressed conditions, and exceptionally high activity when induced with methanol (Cereghino and Cregg, 2000). However, the toxicity and flammability of methanol can be of concern as well as the increased oxygen consumption and heat generation of induced high-cell density cultures, necessitating adapted cultivation procedures (Looser et al., 2015). *pGAP* enables constitutive expression at very similar levels to *pAOX1*, reducing process time and handling complexity (Waterham et al., 1997a). However, *pGAP* is not suitable for the expression of host-toxic products, since production and growth phase cannot be decoupled. Variants of both *pAOX1* and *pGAP* with adjusted transcriptional activity have been developed to enable fine-tuned expression experiments (Hartner et al., 2008; Qin et al., 2011). In addition, many new promoters with different regulatory properties and expression strengths have been discovered and applied. They include alternative, methanol inducible promoters of the MUT pathway (Shen et al., 1998; Tschopp et al., 1987; Vogl et al., 2016) as well as other constitutive or repressible promoters (Moreira de Almeida et al., 2005; Stadlmayr et al., 2010).

P. pastoris exhibits a high genetic accessibility. Since its first discovery, scientists have applied various approaches to shape *P. pastoris* towards their needs. While random mutagenesis and subsequent screening procedures were used in the beginning (Liu et al., 1992), selectable markers (Lin Cereghino et al., 2001) and more sophisticated genetic engineering tools like the Cre-Lox recombinase system (Pan et al., 2011) were established over the years. Applying these techniques, genetic and metabolic engineering projects of different scopes have been realized. However, the aforementioned challenges regarding clonal variability and NHEJ off-target integration events complicated genetic engineering projects of higher complexity. Therefore, improvements to the genetic tractability are an ongoing project. In tandem, the repertoire of integrative and episomal vectors requires expansion to facilitate further development of synthetic biology methods in *P. pastoris* (Kelwick et al., 2014).

The commonly used *P. pastoris* strains were genome sequenced between 2009 and 2011 (De Schutter et al., 2009; Küberl et al., 2011; Mattanovich et al., 2009). In the following years, multiple whole genome transcriptomics (Dragosits et al., 2010; Hesketh et al., 2013; Liang et al., 2012), proteomics (Baumann et al., 2010; Dragosits et al., 2009) and metabolomics (Carnicer et al., 2012; Heyland et al., 2011) studies were carried out. In combination with the detailed characterization of the peroxisome (Wriessnegger et al., 2007) and cellular physiology under protein production conditions (Puxbaum et al., 2015), a good basis for *P. pastoris* systems biology was created. Building on these insights, the first generation of genome-scale metabolic models (GEM) was created (Caspeta et al., 2012; Chung et al., 2010; Sohn et al., 2010). However, the incomplete nature of the available genome data and its annotation was cited as a factor holding back systems biology of *P. pastoris* (Dikicioglu et al., 2014).

In this review, we highlight recent advancements to establish *P. pastoris* not only as a recombinant protein production host but as an entire microbial cell factory. Its capability to synthesize various value-added metabolites and to be genetically engineered in a model-based approach have been successfully demonstrated in recent years. The ease of expressing heterologous proteins of bacterial to human origin in *P. pastoris* ought to make it ideal for transferring metabolic pathways from organisms that produce interesting metabolites, but have other disadvantages like difficulties to be cultivated or genetic inaccessibility. In the following sections the newest developments to improve existing advantages of *P. pastoris*, and ways of circumventing drawbacks, are detailed and put into the context of establishing systems metabolic engineering in this non-conventional yeast.