# Established tools and emerging trends for the production of recombinant proteins and metabolites in *Pichia pastoris*

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## Abstract

Besides bakers' yeast, the methylotrophic yeast *Komagataella phaffii* (also known as *Pichia pastoris*) has been developed into the most popular yeast cell factory for the production of heterologous proteins. Strong promoters, stable genetic constructs and a growing collection of freely available strains, tools and protocols have boosted this development equally as thorough genetic and cell biological characterization. This review provides an overview of state-of-the-art tools and techniques for working with *P. pastoris*, as well as guidelines for the production of recombinant proteins with a focus on small scale production for biochemical studies and protein characterization. The growing applications of *P. pastoris* for *in vivo* biotransformation and metabolic pathway engineering for the production of bulk and specialty chemicals are highlighted as well.

## Introduction

Methylotrophic yeasts raised the interest of biotechnologists first for their ability to grow on methanol as the only carbon and energy source, thus promising the production of cheap protein rich biomass for animal and human nutrition (1). The oil crisis in the 1970s interrupted these plans, however, triggered the development of these yeasts as platforms for the production of heterologous proteins. Among them, *Komagataella phaffii* (formerly known as *Pichia pastoris*) stands out by its wide application in research labs and industrial production. More recently, *P. pastoris* was also employed for the production of metabolites by metabolic engineering. A well-annotated genome sequence (2) as well as genome editing tools and collections of synthetic biology parts and devices make *P. pastoris* a promising chassis for synthetic biology applications (3).

The first yeast strain of what is known today as the *Komagataella* genus was isolated in 1920 by Alexandre Guillermond from a wounded horse chestnut tree and described as *Zygosaccharomyces pastori* (4). Further strains were described as *Pichia pastoris* by Herman Phaff, isolated mainly from oak and pine trees of Southwest United States (5). Based on ribosomal gene sequences *P. pastoris* was further allocated to a new genus, *Komagataella*, and split into two species. With new isolates found over the following years *Komagataella* comprises seven species today (6). The strains used in biotechnology however are usually still all referred to as *Pichia pastoris* which will be used as a synonym for *Komagataella spp*. in the following as well.

## Methylotrophy

Methylotrophy, i.e. the ability to use methanol and similar one-carbon molecules as carbon and energy source, has developed several times among bacteria and yeasts. Methylotrophic yeasts are phylogenetically related and comprise species of the genera *Komagataella*, *Ogataea*, and some *Candida* sp. (7). They share the specific metabolism, first oxidizing methanol by alcohol oxidases (AOX) to formaldehyde, which is further assimilated by dihydroxyacetone synthase (DAS) via the xylulose monophosphate cycle (**Figure 1**). Due to the low specific activity of these enzymes extraordinarily high expression levels are required, so that the promoters regulating *AOX1* and the two *DAS* genes are stronger than any other metabolic gene promoters of *K. phaffii*. The entire xylulose monophosphate cycle is localized in peroxisomes which probably increases its efficiency by metabolic channeling and shielding

the cytosol from toxic intermediates like hydrogen peroxide (8, 9). In three interconnected cyclic pathways 3 xylulose-5-phosphate molecules are regenerated while one glyceraldehyde-3-phosphate is built as a precursor for biomass formation (**Figure 1**). This pathway shares surprising similarity to the Calvin-Benson-Bessham cycle of $CO_2$ assimilation (9). Besides this assimilatory pathway, energy is produced by oxidation of formaldehyde to $CO_2$ via formate, yielding cytosolic NADH which is both used as reduction equivalent and channeled into the respiratory chain to provide ATP.

Methylotrophy was first employed in biotechnology for the production of yeast biomass from natural gas for food and feed applications (1), but soon also considered useful for recombinant protein production because of the strong and regulated promoters, mainly the *AOX1* promoter of *P. pastoris* (10). In a standard setup, methanol is used both as the carbon and energy source and as the inducer of heterologous gene expression. To this end, three different methanol utilization (Mut) phenotypes are employed based on the presence or deletion of the two alcohol oxidase genes. The wild type with both *AOX1* and *AOX2* active is called $Mut^+$, while deletion of the main form, *AOX1*, leads to the $Mut^S$ (methanol utilization slow) phenotype, and strains deleted in both *AOX* genes are called $Mut^-$. $Mut^S$ strains are often employed in protein production while $Mut^-$ strains are considered less suitable based on the assumption that they lack any energy supply from methanol, and also do not provide for formaldehyde and formate which are considered to play a major role in the induction of methanol utilization genes (11). Zavec et al. (12) showed however that also $Mut^-$ strains consume methanol and can serve as a platform for the production of proteins. The same authors demonstrated recently that native alcohol dehydrogenase (Adh2) of *P. pastoris* is responsible for methanol oxidation in $Mut^-$ strains (13).

## Tools & Techniques

From strain construction until bioprocess optimization for recombinant production in any expression system, several product-specific aspects need to be considered. This is where the well-established and constantly expanding set of tools and techniques available for strain and bioprocess engineering (**Figure 2**) contributes in making *P. pastoris* a highly efficient expression system (14, 15).

**Design of the expression construct**

*P. pastoris* offers a range of well-characterized constitutive and inducible promoters to choose from. The constitutive promoter $P_{GAP}$ has frequently been used in large scale production of recombinant proteins in *P. pastoris* (16). Other strong constitutive promoters include $P_{TEF1}$ and $P_{GCW14}$ (17). However, constitutive promoters are not ideal for recombinant proteins that are toxic to the cells. In terms of inducible promoters, most commonly the methylotrophic feature of *P. pastoris* is harnessed by driving the expression of recombinant genes under the influence of the methanol-inducible promoter of the alcohol oxidase I (*AOX1*) gene (18). Other strong methanol-based promoters include $P_{FLD1}$ (also induced by methylamine), $P_{DAS}$ and $P_{CAT1}$ (also induced by glycerol and de-repressed under carbon source limitation) (17). While methanol-based promoters provide strong induction, their disadvantage in terms of toxicity of methanol has led to extensive research for the discovery of alternative, methanol-independent expression systems. On one hand, cell engineering has been successfully applied to develop methanol-independent $P_{AOX1}$ and $P_{DAS1}$ host systems (19, 20). Hartner *et al.* created a library of several $P_{AOX1}$ variants, by which they not only identified variants with activities higher than the native promoter upon methanol induction, but also identified some variants which demonstrated strong methanol-independent activity in de-repressing conditions (21). On the other hand, several methanol-independent promoters were identified, including $P_{GTH1}$ (induced in glucose-limited conditions), $P_{THI11}$ (thiamine repressed) and $P_{ADH3}$ (ethanol-induced) (22-24). Orthologous promoters have also been successfully used, for example, the promoter of the methanol oxidase gene from *Hansenula polymorpha* ($P_{MOX}$) (25, 26). Promoter engineering has been applied to some of these native promoters as effective tool for engineering existing promoters for better performance (27-30). Additionally, bidirectional promoters have been engineered which can offer an advantage when co-expression of multiple genes is necessary (31).

Secretion of the recombinant product can simplify the downstream purification steps and therefore is often desirable. Signal peptides play a very important role in efficiently driving the secretion of the recombinant protein. While the secretion signal of the *Saccharomyces cerevisiae* α-factor mating pheromone is most commonly used to drive the secretion of recombinant proteins in *P. pastoris*, there have been instances where the native signal peptide of the protein or other endogenous signal peptides were shown to have similar or

even higher efficiencies (32, 33). Additionally, higher yields of recombinant proteins have been demonstrated using modified versions of the *S. cerevisiae* α-mating factor secretion signal (34). However, selection of signal peptide can be protein-dependent and therefore, testing multiple signal peptides can lead to higher yields (35).

In addition to transcriptional termination, terminator regions can also influence the level of expression. The toolbox of terminators in *P. pastoris* was quite small, consisting of only 20 terminators with not much variation in expression between them (36). Only recently, a catalog of 72 endogenous, heterogeneous and synthetic terminators was developed and characterized, within which a tunability of 17-fold was observed (37).

**Selection of vector and background strain**

While there have been some studies towards the generation of circular plasmids with autonomously replicating sequences (*ARS*) for stable recombinant expression in *P. pastoris*, genomic integration vector systems are most commonly used (38, 39). A selection of vector systems is commercially available with a choice of antibiotic or auxotrophic selection markers (40, 41). Additionally, there are vectors based on the Golden*Pi*CS modular cloning system which enable single reaction assembly of selected promoters, terminators, resistance cassettes and genomic integration loci (36). For selection of a background strain, one gets to choose from various wild-type and auxotrophic strains, strains with different methanol-utilization phenotypes ($Mut^+$, $Mut^S$ & $Mut^-$), protease-deficient strains and glycol-engineered strains (42).

**Molecular biology methods**

Several well-established molecular biology techniques in *P. pastoris* aid in speeding up the strain generation process (**Figure 2**). The modular cloning toolbox of Golden*Pi*CS, mentioned earlier, allows one-step assembly of multiple expression cassettes into a single vector, which can then be linearized and integrated into the genome of the host strain. This method requires the use of selection markers, which can be exploited to increase the copy number of the recombinant gene construct by increasing the selection pressure. This can be an advantage since increased gene dosage is often associated with increase in protein titers (15, 43, 44). In case of antibiotic resistance markers, there is a possibility for removal of the resistance gene post selection of positive clones by transient expression of Cre recombinase,

however, this is not applicable if multicopy strains are intended (45). Recent development and fine-tuning of the CRISPR/Cas9 technology in *P. pastoris* allows for marker free integration of expression cassettes (46-48). While the technology still has its limitations, a large amount of research has been directed in improving the technology leading to higher efficiencies, possibility of multiple genomic integrations as well as application of deactivated Cas9 (dCas9) for targeted gene interference (49).

**Strain selection & bioprocess optimization**

The final steps in strain development comprise evaluation of generated strains for final clone selection and bioprocess optimization (**Figure 2**). Strain evaluation, often also referred to as "screening", is usually done in shake-flasks or in high-throughput microtiter plate cultivation. Guidelines for such screenings are given in **Box 1**. To find suitable production clones, screening of at least 20-40 clones is recommended. Depending on the protein of interest and the desired product titer screening of several hundred clones may be required, which makes high-throughput screening methods desirable. While on one hand it is possible to mimic batch or fed-batch fermentation conditions and select optimum clones, bioprocess optimization is usually not possible in shake-flasks/microtiter plates. In that respect mini/microscale cultivations or microfluidics and Lab-on-Chip based technologies provide alternative screening platforms that also integrate bioprocess optimization, making scale-up more convenient (50-53).

**Strain engineering to improve protein production**

Several studies report strategies on how to enhance recombinant protein production and secretion in *P. pastoris* (summarized in 54, 55). Such cell engineering approaches are mostly not applied for initial characterization, unless a specific bottleneck is observed. Examples are mainly the avoidance of proteolytic degradation through use of strains deficient in the major cellular proteases such as *pep4* deficient strains or the co-expression of certain chaperones, especially protein disulfide isomerase (55, 56). Some proteins such as cytochrome P450s or heme-containing proteins require co-factors that either need to be added externally or produced by the cells (57).

### Systems biology

While a lot of advances has been made regarding the tools and techniques for strain engineering in *P. pastoris*, development of systems biology tools is steadily gaining importance. For example, genomic and proteomic studies have been applied to some recombinant strains under conditions of recombinant protein production (54). Metabolic flux analysis (MFA) has been carried out to understand the metabolic impact of recombinant protein production in *P. pastoris* (58). Extensive MFA studies have been performed to compare metabolite concentrations under different cultivation conditions (9, 59, 60).

Genome scale metabolic models (GEMs) have the potential to identify engineering targets to alleviate metabolic burdens as well as to develop efficient bioprocess strategies. Several GEMs have been available for *P. pastoris* since 2010 (61-65). Additionally, efforts have been directed towards refining or upgrading existing GEMs, for e.g. by incorporating native and humanized N-glycosylation pathways for production of glycoproteins (66), improving biomass synthesis equations allowing improved prediction capabilities over a wide range of substrates (67) or including additional reaction pathways (68). Thus, systems-level studies are now being incorporated into the strain engineering and improvement workflow.

## *Pichia pastoris* as a host for recombinant protein production

### *P. pastoris* produced recombinant proteins for biomedical and industrial applications

Since the late 1990s *P. pastoris* has been used to produce proteins for biopharmaceutical, industrial and diagnostic applications. Commercialized products include insulin, growth factors, interferon, and subunit vaccines such as hepatitis B surface antigen, and several others including peptides and antibody fragments (especially single chain antibody fragments and nanobodies) are in the clinical pipeline (56, 69, 70). Furthermore, industrial enzymes such as phospholipase C and phytase are produced using *P. pastoris* as host (71, 72). *P. pastoris* is also described to be a preferred expression system to prepare several other recombinant subunit antigens against human and animal pathogens (70, 73), e.g. brucellosis subunit vaccine (74), Zika virus envelope domain III (75), influenza hemagglutinin and neuraminidase (69), and lately also Sars-CoV-2 spike antigen (76).

Very recently, *P. pastoris* has entered into another emerging market, as soy leghemoglobin produced in *P. pastoris* was approved as a flavor component of cultured meat by the FDA (77, 78).

While virus antigens are often produced as intracellular virus like particles (VLPs) (73) or self-assembling nanoparticles (69), most proteins produced in *P. pastoris* are secreted (56, 70). This allows for a reduced number of downstream processing steps during purification compared to intracellular production (56). As several proteins secreted by *P. pastoris* have received a GRAS notice by the FDA (77), it is even possible to directly use the culture supernatant containing the secreted protein of interest in some cases such as in animal feed applications (79).

**Production of recombinant proteins for structural and biophysical analysis**

Apart from being an important host for commercial protein production purposes, a high number of published studies deal with using *P. pastoris* to provide a wide variety of heterologous proteins and mutants thereof for biochemical characterization such as enzyme activity or for structural and biophysical studies. Often these studies are the first steps for later utilization of enzymes. For example, *P. pastoris* was used to determine 1.7-Å resolution crystal structures and to identify the substrate specificities and the catalytically active sites of several *Aspergillus* rutinosidases, which are potential catalysts for flavonoid compounds with nutritional value (80, 81). Substrate specificity and crystal structures of several plant and fungal carbohydrate active enzymes have been elucidated by expressing them in *P. pastoris*. Heterologous expression of plant glycosyltransferases did not only result in the production of mannan and glucomannan in *P. pastoris*, but additionally provided important insights into the yet unresolved biosynthesis of these polysaccharides in plants (82, 83). Site-directed mutagenesis was also carried out for proteins of biomedical interest, e.g. to study immunogenic residues of the buckwheat allergen (84), to increase efficacy and stability of ocriplasmin (85) or to strengthen the affinity of a scFv to its target and should be even more facilitated by the high-throughput cloning tools available for *P. pastoris* now (see section Tools & Techniques).

Membrane proteins (MP) are a particularly challenging class of proteins that have been produced with a quite high rate of success in *P. pastoris* (86, 87). Several efforts have been

undertaken to improve the expression levels and purification of MPs. Common cell engineering strategies and tools for MP production in yeast were summarized by Byrne, 2015 and Routledge *et al.* 2016 (86, 88). Recently, a novel procedure was devised in which misfolding and aggregation of integral MPs is avoided by directly solubilizing them from protoplasts instead of crude membrane preparations (89). Nanodisc reconstitution was successfully applied to generate sufficient amounts of purified channelrhodopsins for biophysical characterization (90). The human tetraspanin CD81 expressed in *P. pastoris* could be efficiently solubilized and purified within a lipid environment by using styrene-maleic anhydride co-polymers, providing a platform to study the influence of protein-lipid interactions of tetraspanins (91).

Crystal structures and biophysical characterization of human amino acid transporters, aquaporins and several human G protein coupled receptors (GPCRs) expressed in *P. pastoris* contributed significantly to our understanding of their substrate transport dynamics (87, 92-94). The recent elucidation of the mating pathway in *P. pastoris* together with its high expression capacity of GPCRs, so far mainly exploited for structural studies, paves the way to perform *in vivo* GPCR signaling studies as described for *S. cerevisiae* (95, 96).

*P. pastoris* has been used since the 1990s to produce isotope-labeled proteins for NMR-based structural analysis, and it is the preferred yeast host for this powerful structural biology technique (87, 94). NMR spectroscopy is well suited to study protein-ligand interactions and dynamics and to guide ligand design, however, for such purposes incorporation of sophisticated isotopic labels is often required. Zhang, 2020 provided a collection of suitable labeling protocols and summarized the most recent advances made in *P. pastoris* (94). Labeling strategies include global or selective $^{13}C$ $^{15}N$-labeling, $^{19}F$-labeling, deuterium labeling for improved resolution of spectra, and methyl-labeling of valine, leucine or isoleucine for even more improved sensitivity of spectra of high-molecular-weight proteins or complexes. Site-specific methyl-labeling in a deuterated background permitted sensitive methyl-TROSY experiments in *P. pastoris* that were successfully conducted to elucidate structure-function relationships of several human GPCRs of biomedical relevance (87). On the other hand, selenomethionine labeling might be applied to improve X-ray crystallography (97). For all of the latter approaches, uptake of labeled amino acid(s) might be limiting, thus cell engineering provides a promising strategy for improving labeling

efficiencies (94, 98). Cell-free expression systems recently established also for *P. pastoris* are another alternative especially for the incorporation of otherwise cytotoxic selenomethionine (99).

**Design of culture conditions for small scale protein production**

While projects aiming at producing a certain protein or compound usually required large scale cultivation in bioreactors, studies aiming at protein characterization are often (at least initially) performed in small scale such as shake flasks or deep well plates (100). Guidelines for selecting suitable producer clones are given in **Box 1**.

Often, substantial efforts are invested to improve the cultivation conditions for a certain protein of interest. Based on our experience we suggest investigating the impact of pH, which should not be too close to the isoelectric point of the protein to avoid precipitation, and may as well impact product quality and stability through proteolysis. Many researchers investigate methanol concentration, with the outcome that in most cases addition of 0.5-1% methanol twice a day works best. Usually higher cell density and higher product titers are obtained in complex medium containing yeast extract and peptone or casamino acids, however, proteolytic activity (probably due to cell lysis) might be observed. For secretory proteins, growth-limiting carbon supply (e.g. by enzyme-mediated glucose release simulating a fed batch regime) leads to good product titers and high product quality in our experience (49, 101), and works comparably well for both complex and synthetic media. This cultivation strategy is also well suited for the above described amino acid labeling strategies where the use of minimal medium, with or without deuteration, is required, and helps to overcome the previously reported limit of low protein amounts.

## Metabolite production by whole-cell biotransformation and fermentation bioprocesses

Although *P. pastoris* has been developed primarily as a cell factory for recombinant protein production, its potential for metabolite production purposes has received increasing attention over the past 15 years, likely boosted by the increasing physiological knowledge base and synthetic biology tools availability (see excellent reviews by (102-104)). The use of *P. pastoris* has been demonstrated for the production of diverse chemical compounds,

particularly for complex metabolites such as plant secondary metabolites (polyketides, terpenoids, isoflavonoids) and other drug metabolites (e.g. steroids, sphingolipids) as well as, to a much lesser extent, for bulk chemicals such as organic acids, biofuels or biopolymers (**Figure 3**). Nonetheless, most of these examples are proof-of-principle studies reporting titers, yields and productivities far from industrially attractive metrics.

Two major metabolite production approaches have been explored, namely biotransformation (biocatalysis) and fermentation bioprocesses. Whereas in fermentations the products are synthesized from carbon/nitrogen substrates via the host cells' native or engineered metabolism, in biotransformations, cell growth and product synthesis phase are separated, i.e. biotransformations are typically performed by resting cells, which convert substrates/precursors to the desired products. Generally, biotransformations are catalyzed by intracellular enzymes, although cell surface display of enzymes for biotransformation purposes has also been demonstrated.

*P. pastoris* presents several *a priori* physiological advantages and specific metabolic traits that make this yeast particularly interesting for whole-cell biocatalytic systems. i) As Crabtree negative yeast, high cell densities, that is, high amounts of biocatalyst, can be easily obtained from bioreactor cultures using cheap substrates; also, *P. pastoris* shows higher resistance to harsh process conditions such as low pH compared to other cell factories such as *Escherichia coli*. ii) The native alcohol oxidase (Aox), a peroxisomal enzyme with relative low substrate specificity, can catalyze the oxidation of many short-chain alcohols to the respective aldehydes using oxygen as electron acceptor, generating hydrogen peroxide as a toxic by-product. Notably, *P. pastoris* also synthesizes large amounts of catalase in the peroxisomes together with Aox during methylotrophic growth, thereby providing an interesting system for oxidase-catalase-based reactions (104). This concept can be further expanded by, e.g. targeting heterologous oxidases such as D-amino acid oxidases (DAO) to the peroxisome, providing a system for α-keto acids production from α-amino acid substrates and resolution of racemic mixtures of amino acids (105). iii) The native methanol dissimilation pathway, which oxidizes formaldehyde stepwise to formate and $CO_2$ by two $NAD^+$-dependent dehydrogenases has been exploited as a natural NADH regeneration system for reduction reactions. Moreover, such endogenous system has been specifically optimized for whole-cell biotransformation purposes by overexpressing the formaldehyde dehydrogenase encoding gene (FLD) and disrupting the DAS genes, key genes in the

competing methanol assimilatory pathway (106, 107). Also, NADPH regeneration engineered systems or process strategies have been proposed (104). For instance, Tang and co-workers (108) have used glucose as a co-substrate for NADPH synthesis to improve NADPH-dependent synthesis of the steroid boldenone, an androgenic–anabolic steroid and a testosterone derivative. iv) *P. pastoris* has shown a high aptitude for expression of membrane bound proteins like cytochrome P450 monooxygenases (CYP) and reductases (CPR). v) Under methanol-growing conditions, peroxisomes largely proliferate which offers the possibility to compartmentalize synthetic metabolic pathways in this organelle as a metabolic engineering strategy (103).

*P. pastoris* has also been used in whole-cell biotransformations requiring ATP such as the synthesis of dipeptides and tripeptides, as well as in other reactions not dependent of cofactors, e.g. hydrolysis, carbon-carbon bond formation reactions, and further synthesis reactions. Some recent exemplary studies include the production of the dipeptides (109), synthesis of phospholipids (110), and the use of transketolase-overproducing strains to catalyze asymmetric carbon-carbon bond formation reactions, e.g. the production of L-erythrulose from prochiral substrates (111) or, strains combining the overexpression of transketolase and ω-transaminases encoding genes for chiral amino-alcohols synthesis (112, 113).

Despite the fact *P. pastoris* presents several features such as an industrially well-established fermentation technology and the ability to grow on renewable feed stocks like glycerol and methanol that are more reduced than glucose, it remains largely unexploited as a cell factory for bulk/platform chemicals and biofuels production (102, 103). For instance, conversion of glycerol into L-lactic acid has been demonstrated by the expression of a lactate dehydrogenase gene from *Bos taurus* (114). Additional metabolic engineering of this strain investigated the impact of deletion of the pyruvate decarboxylase gene, aiming at reducing acetic acid formation and consequently obtaining higher lactic acid titers. This resulted in a yield of 67% L-lactic acid and 20% arabitol as a by-product in glycerol batches with oxygen limitation (115). Further disruption of the arabitol dehydrogenase encoding gene has been recently reported by the same group (116), obtaining an increase of 20% in lactic acid and a 50% reduction in arabitol yields in chemostat cultures. Also recently, the production of D-lactic acid from methanol using an engineered *P. pastoris* strain expressing multiple copies of

the D-lactate dehydrogenase gene from *Leuconostoc mesenteroides* has been reported (117). Similarly, production of an increasing number of platform chemicals has also been demonstrated, typically at shake flask or small bioreactor scale, including isobutanol and isobutyl acetate (118), isopentanol (3-Methyl-1-butanol) (119), and 2,3-butanediol (120). Moreover, the potential of *P. pastoris* to produce malic acid from methanol has been lately explored through different metabolic engineering strategies, obtaining up to about 2.8 g/L of malic acid in shake flask cultures (121).

So far, these novel developments to metabolically engineer *P. pastoris* for metabolite production have generally relied on classical genetic tools (promoters, markers etc.) and conventional strains that were originally optimized for recombinant protein production. Despite enormous advances in systems and synthetic biology tools in recent years, significant challenges in genome engineering remain compared to *Saccharomyces cerevisiae*, e.g. generally lower efficiency of currently available CRISPR and homologous recombination based methods (122). The incorporation of new-generation synthetic biology tools should speed up development of metabolite production strains beyond mere demonstration/proof-of-concept. To this end, the design of robust chassis strains supporting efficient conversion of alternative carbon substrates/energy sources, for e.g., $CO_2$ (123), acetate (124) or cellulose/cellobiose (125) to key metabolic precursors e.g. acetyl-CoA, farnesyl pyrophosphate, malonyl-CoA (**Figure 3**) will be key in the forthcoming years.

## Summary points

- Methylotrophy and the strong methanol regulated promoters were the key drivers to develop *P. pastoris* to a protein production host.
- Early free access to strains and vectors has promoted research on *P. pastoris* enormously.
- The development of tools and parts for *P. pastoris* make it an important synthetic biology chassis.
- A wide range of available molecular and synthetic biology toolkits contributes in developing a simple workflow from strain design to bioprocess optimization.
- The efficient native one-carbon metabolism offers promising perspectives for a single-carbon (methanol, formate, $CO_2$) based bioeconomy.

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## Figure Legends

**Figure 1: Metabolic pathways of methanol utilization in *P. pastoris*.** After oxidation of methanol to formaldehyde assimilation is achieved in the xylulose-5-phosphate cycle, indicated in green. A byproduct of methanol oxidation is hydrogen peroxide, which is further detoxified by catalase. These reactions, as well as the Xu5P cycle, are localized in peroxisomes. Formaldehyde is also dissimilated to CO2 in three cytosolic reactions, indicated in blue. The produced NADH serves as reduction equivalents and for ATP production. Assimilation of three formaldehyde molecules leads to the release of one molecule of glyceraldehyde-3-phosphate from peroxisomes which serves for biomass growth.

GSH: reduced glutathione; GS-CH2OH: S-(hydroxymethyl)glutathione; GS-CHO: S-formylglutathione; Cat1: catalase; Fld1: formaldehyde dehydrogenase; Fgh1: S-formylglutathione hydrolase; Fdh1: formate dehydrogenase; Aox1/2: alcohol oxidase 1 and 2; Das1/2: dihydroxyacetone synthase 1 and 2; Dak2: dihydroxyacetone kinase; Tpi1: triosephosphate isomerase; Fba1-2: fructose 1,6-bisphosphate aldolase; Fbp1: fructose 1,6-bisphosphatase; Shb17: sedoheptulose 1,7-bisphosphatase; Rki1-2: Ribose 5-phosphate ketol-isomerase; Rpe1-2: D-ribulose 5-phosphate 3-epimerase.

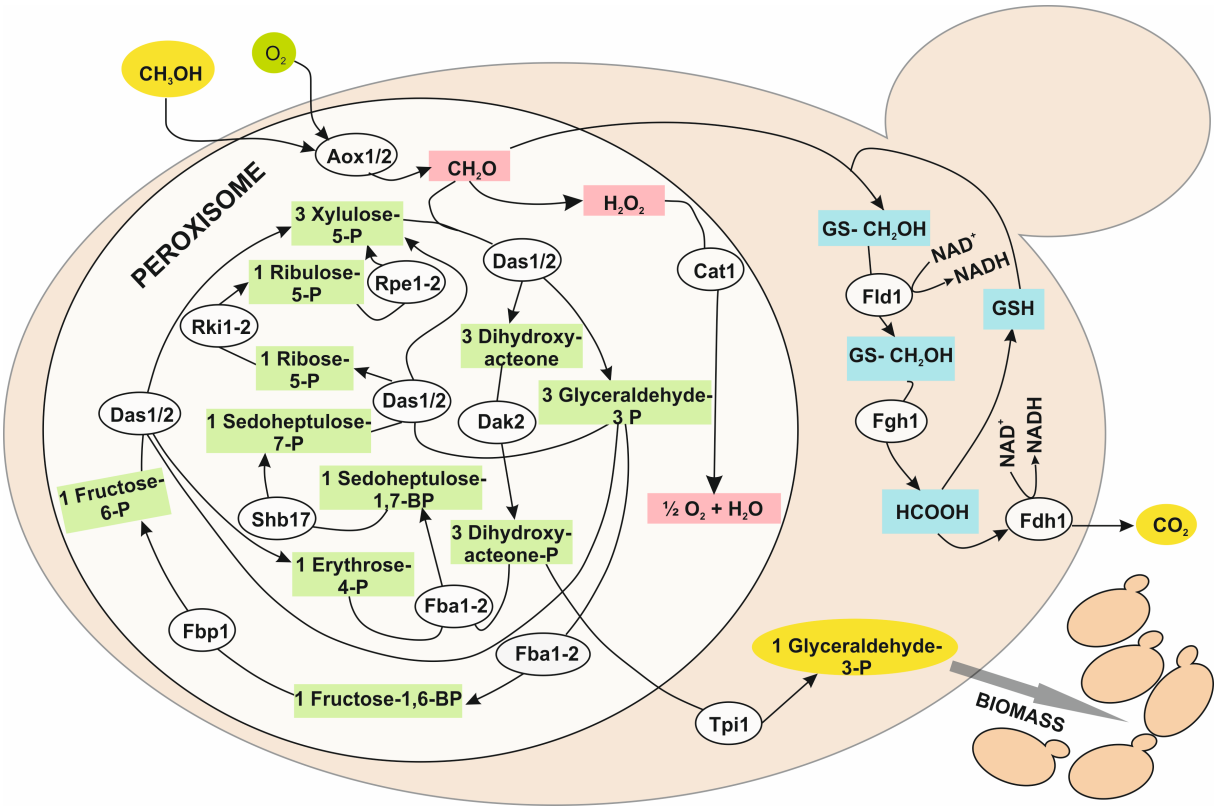


**Figure 2: Workflow from construct design to bioprocess optimization and strain improvement for recombinant protein production in *P. pastoris*.** A. After selection of the different modules, the expression cassette can be constructed using GoldenPiCS. There is a possibility to integrate the expression construct in the recombinant strain either with selection marker by direct plasmid linearization and integration or marker-free by CRISPR/Cas9-based homology directed recombination. B. An optimum strain background depending on the recombinant protein can be selected. C. Well-established transformation protocols are available for generating the recombinant strain. D. Different screening strategies enable evaluation of a large number of clones resulting in faster clone selection. Lab-on-Chip technology also allows bioprocess optimization at this stage. E. Final clones can be cultivated in bioreactors for producing high titers of the recombinant protein. F. Data obtained during bioreactor cultivations combined with systems biology tools can be used to identify further cell engineering targets for strain improvement

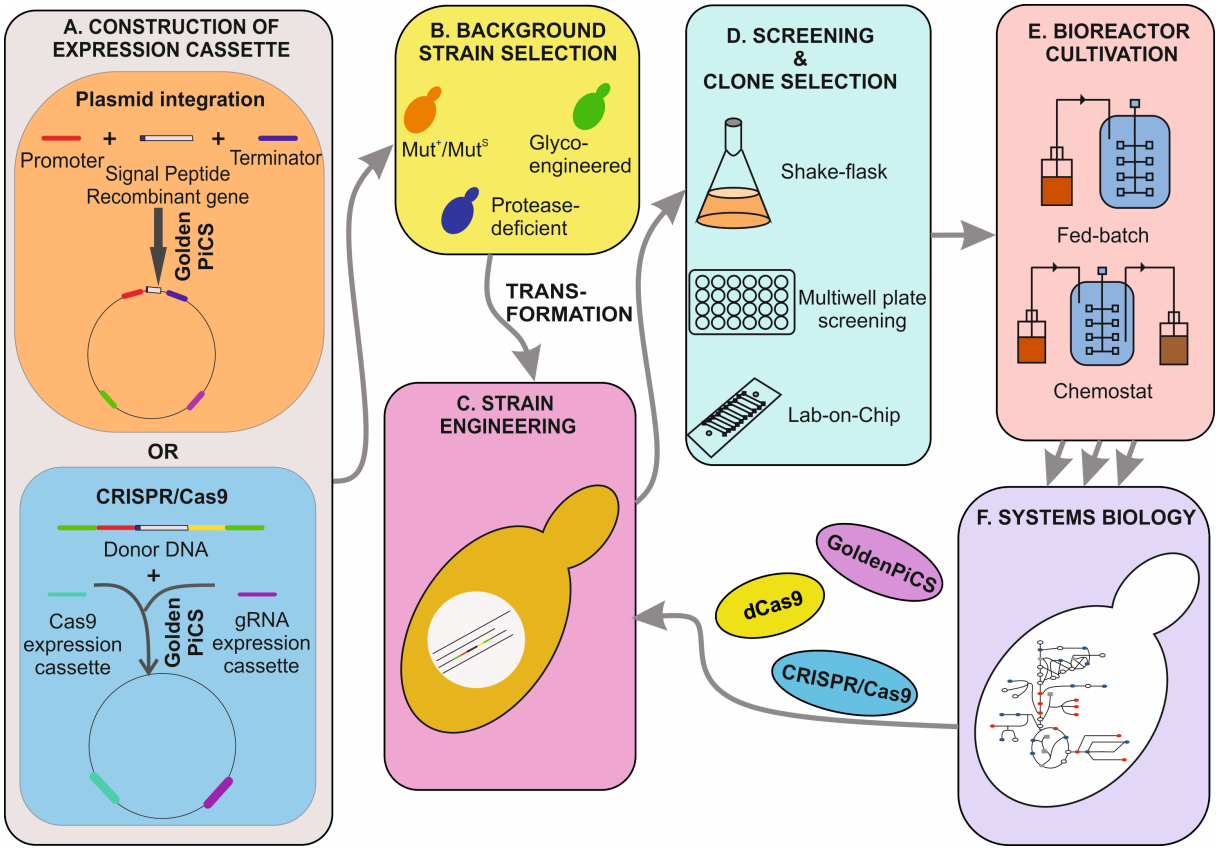
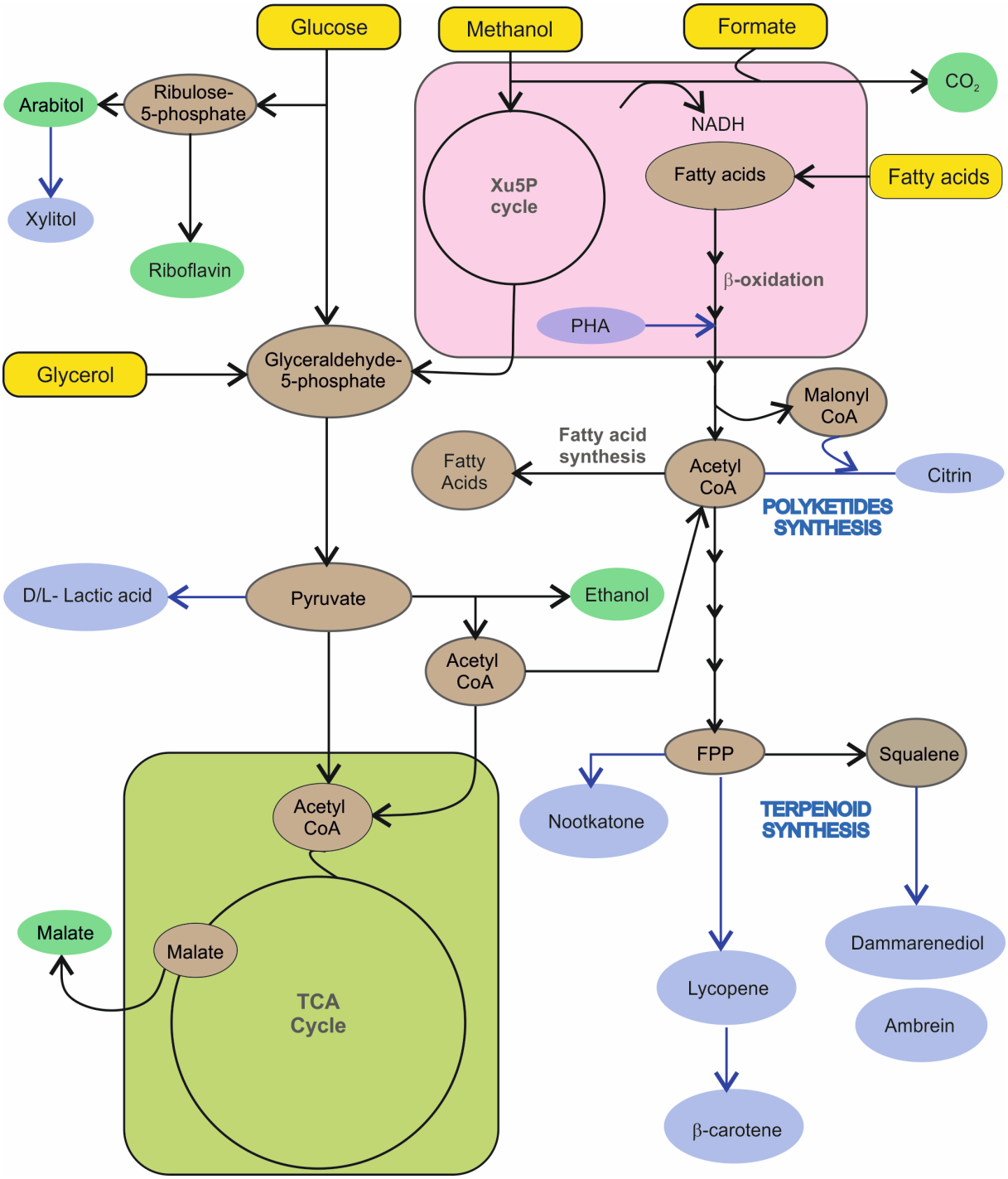


**Figure 3: Metabolites overproduced in *P. pastoris*.** Major pathways towards key metabolic precursors or metabolic nodes (in brown) and products already made by *P. pastoris* (green) are depicted in black. Heterologous pathways and metabolites are depicted in blue. Relevant substrates that *P. pastoris* can naturally assimilate as carbon and/or energy source are shown in yellow.



**Box1: Recommendations for selection of a producer strain**

Guidelines how to select strains for biochemical studies or protein production purposes.

1. After transformation, plate aliquots on a suitable selection medium e.g. YPD containing 100 µg/mL Zeocin (in case your vector has the Zeo resistance cassette). As strains with higher gene copy number are often correlated with higher product titers, you might also plate aliquots at a higher Zeocin concentration e.g. 250 µg/mL, 500 µg/mL or 1000 µg/mL.
2. After restreaking, use 20-40 transformants (selected from all Zeocin concentrations) for the initial screening. On average, 5-10% of the clones outperform the others. There may be cases where it is necessary to screen more clones, e.g. when searching for a high production clone of a very difficult to produce protein.
3. Initial screening can be performed in 24 deep well plates, 96 deep well plates or any other suitable format depending on the volume needed for quantification and analysis.
4. Select the pH of the cultivation medium according to your protein of interest (avoid being close to the isoelectric point or a pH where degradation might occur). In most cases, the starting pH of the medium is between 5.0-7.0, and a suitable buffer (e.g. phosphate buffer, MES (2-(N-morpholino)ethanesulfonic acid), citrate) is used to stabilize the pH during cell growth.
5. Prepare cryo stocks of your strains (e.g. 1 mL of selective overnight culture + 10% glycerol and freeze at -70°C) early during screening. For further experiments, the preparation of working cell banks (WCBs) from this stock is recommended to avoid repeated freeze/thaw cycles of the stock.
6. Rescreen the 3-5 best or most-interesting clones to evaluate their performance, and select 1 or 2 for further experiments.
7. If optimization of screening conditions is required, we recommend to evaluate different pH values (see above) and cultivation temperatures (e.g. 20-25°C compared to 28-30°C).
8. For inducible expression, e.g. using the *AOX1* promoter, a pre-culture should be performed: overnight culture in selective YP-based medium to inoculate pre-culture on glycerol at low $OD_{600}$ and grow for 20-24 h; inoculate main culture at higher OD (e.g. $OD_{600}$ = 4 or 8) and induce with 0.5% methanol; add 1% methanol approx. every 12 h.

9. As secreted protein titers are also correlated to the achieved biomass concentration, screening conditions often aim at high biomass by using complex medium. However, in some cases such conditions resulting in fast growth are leading to product degradation due to cell lysis, or the strain performance cannot be upscaled to the bioreactor later. In such cases, growing the cells below their maximum specific growth rates by simulating fed-batch like conditions already in screening by using a carbon-release system is recommended.

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