3
Expression Systems: *Pichia pastoris*

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3.1 Introduction

Among the most widespread, popular, effective, and inexpensive microorganisms developed for heterologous expression, *Pichia pastoris* has become a system of choice not only for the production of cytosoluble and industrially relevant proteins, but also for a growing panel of eukaryotic membrane proteins expressed at levels compatible with structural studies. Up to now indeed, more than 150 different representative membrane proteins have been expressed in *P. pastoris* and this has led to the acquisition of high-resolution structures for a dozen of them, making *P. pastoris* one of the most performant heterologous expression system for the structural studies of eukaryotic membrane proteins. This chapter gives a global overview on (i) how the *P. pastoris* system basically operates, (ii) how it performs for the recombinant expression of membrane proteins, and (iii) the different strategies and tips that can be applied to improve the system.

3.2 A (Brief) Summary on the (Long) History of *P. pastoris*

*P. pastoris* is an ascosporous yeast that is naturally present in tree fluxes from European and north American forests [1]. It was first isolated in 1919 in France from the exudate of a chestnut tree [2] and half a century later was described for its ability to use methanol as a sole carbon source [3]. From this time point, the potential of *P. pastoris* for biotechnological applications has been continuously explored both in industry and academia. While its use as a potential source of single-cell protein for animal feed did not meet the expected economic viability [4], this yeast was rapidly recognized as a remarkable production platform for a wide class and number of heterologous proteins, and up to now more than 500 candidates have been successfully recombinantly expressed in this system (reviewed in [5, 6] among others), several of them being biopharmaceuticals already on the market. Recently, phylogenetic analyses based on rRNA sequence
comparisons led to the transfer of *Pichia pastoris* into the *Komagataella* genus [7], and the commonly used biotechnological strains are now classified into two distinct species, *K. pastoris* and *K. phaffii* [8]. The saga of this special yeast, that we will continue to call *P. pastoris* for the sake of simplicity, will be certainly boosted in the coming years after the very recent release of its genome sequence [9]. This new wealth of information will indeed open new possibilities for the engineering of enhanced biotechnological strains.

### 3.3 Introducing *P. pastoris* as a Biotechnological Tool: Its (Extended) Strengths and (Limited) Weaknesses

The success of *P. pastoris* as an efficient protein factory is attributable to a series of advantages related to both its yeast nature and its particular methylotrophic metabolism. This organism indeed presents a short generation time (2 h), grows on very simple and inexpensive media, and is very easy to handle. A comprehensive panel of plasmids and strategies is available for the expression of recombinant genes and genetic manipulation is nearly as straightforward as for *Saccharomyces cerevisiae*. As a methylotroph, *P. pastoris* possesses a peculiar methanol utilization pathway relying on some of the strongest and most tightly regulated known promoters that can be used for very-high-level expression of recombinant genes. In addition, *P. pastoris* can reach very high cell densities (up to 130 g/l dry cell weight [5]), and various fermentation processes and formats have been developed in the industry so that up-scaling protein production is easily achievable [10, 11]. Contrary to *Escherichia coli*, *P. pastoris* is a eukaryotic microorganism capable of complex post-translational modifications including disulfide isomerization, sulfation, phosphorylation, N-terminal acetylation, C-terminal methylation, myristoylation, farnesylation, and glycosylation (reviewed in [12]), which are often very essential for the proper targeting, biological activity, and stability of the expressed recombinant proteins. Regarding glycosylation, which is central for many membrane proteins [13–15], *P. pastoris* has been notably shown to graft shorter and more authentic oligosaccharide chains to proteins than *S. cerevisiae* does [16], and therefore often appeared as a more appropriate system [11]. Finally, $^{15}$N and $^{13}$C isotopic labeling of recombinant proteins for nuclear magnetic resonance or spectrometric studies is also achievable with *P. pastoris*, both in a uniform mode using isotopically enriched nitrogen and carbon sources [17] or more selectively using amino acid isotopes and engineered auxotroph strains [18].

This idyllic description has, however, to be tempered with some drawbacks that prevent *P. pastoris* from becoming an ideal expression system. First, a common characteristic for eukaryotic systems that are efficiently overproducing proteins, an overload of the translocation and folding machineries in *P. pastoris* often creates a stress that triggers the activation of sorting and degradation processes, and results in lowered expression levels and heterogeneity of recombinant proteins [18]. Moreover, if glycosylation processes occur in a fashion acceptable for many
recombinant proteins, *P. pastoris* is not able to graft the complex carbohydrate motifs that are sometimes critical for the functionality of mammalian proteins. In addition, nonhomogeneous N-glycosylation of recombinant proteins is frequently observed, notably in the case of membrane proteins, leading to some degree of heterogeneity [19–22] that can be detrimental in various applications, including structural studies. Recently, several *Pichia* strains have been engineered to generate more complex and more homogeneous N-glycosylations (reviewed in [23]). These strains exhibited protein-dependent but promising outcomes, thereby also demonstrating all the potential and possibilities that could be gained from these genetic engineering approaches.

Another characteristic that can have a direct impact on the expression of mammalian membrane proteins is related to the lipidic composition of yeast membranes that varies significantly from that of higher eukaryotes membranes [24]. As membrane proteins do require specific lipids for their proper functions or for their correct folding and stability, these differences may influence both the expression level and functionality of recombinant membrane proteins. This was notably reported in studies where the absence of cholesterol in *Pichia* membranes was shown to profoundly alter the activity and stability of recombinant membrane proteins [25].

Finally, a secondary but not trivial issue is related to the presence of a significant cell wall surrounding *Pichia* cells that cannot only hinder the secretion of certain proteins [26], but also represents an obstacle for the preparation of membrane-embedded proteins as aggressive disruption methods are needed [27]. Engineered strains with weaker cell walls have been recently developed [26, 28], but none has been reported yet for its use in heterologous expression of membrane proteins and their benefit for a facilitated cell disruption has still to be assayed.

### 3.4 Basics of the *P. pastoris* Expression System

This section is intended at give a global overview on how the system functions for the heterologous expression of proteins in general, before giving specific details on how it performs in particular for membrane proteins (Section 3.5). Further details on the system as well as additional information concerning secreted and/or cytosoluble proteins can be found in several excellent and comprehensive reviews that have been published on the topic [5, 6, 11, 29].

#### 3.4.1 Methanol Utilization Pathway

Together with a small set of methylotrophic yeasts from the *Pichia*, *Komagataella*, *Candida* and *Ogatae* genera, *P. pastoris* has developed a specific metabolism for the utilization of methanol as sole carbon source. Briefly, methanol enters specialized microbodies, the peroxisomes, where it is oxidized by specific oxidases...
that are encoded by the two genes $AOX1$ and $AOX2$ to generate formaldehyde and hydrogen peroxide. While the latter compound is decomposed to water and molecular oxygen by a peroxisomal catalase, formaldehyde leaves the peroxisome to enter both the cytosolic dissimilatory pathway to yield energy and the assimilatory pathway for generation of biomass [30]. The genes encoding the specific enzymes related to this peculiar metabolism are repressed when cells are grown on nonmethanol carbon sources (glucose, glycerol, ethanol, etc.) and are dramatically induced in presence of methanol; alcohol oxidases representing as high as 30% of the total soluble protein content. These enzymes are thus very tightly regulated and their promoters represent ideal components to be used for recombinant expression – the basis for the development of the $P. pastoris$ expression system.

3.4.2 Host Strains and Plasmids

The principal strains used for recombinant expression derived either from the NRRL Y-11430 (Northern Regional Research Laboratories, Peoria, IL) or the NRRL Y-48124 (Invitrogen expression kit) strains, both being from the $K. phaffii$ type [8]. This limited number of strains is listed in Table 3.1. They mainly differ in their auxotrophic behavior, principally relying on a histidinol dehydrogenase deficiency ($his4$), allowing, upon transformation, for the positive selection of recombinant expression vectors. Some of them bear additional deficiencies in endogenous proteases (SMD series); others were recently engineered for their capacity in performing “human-like” N-glycosylations [23].

Expression vectors (Table 3.2) are built on a classical $E. coli$/yeast shuttle model.

Table 3.1 Most commonly used strains of $P. pastoris$.

| Strain | Genotype | Phenotype |
|--------|----------|-----------|
| NRRL a) Y-11430 | wild-type | Mut$^+$ |
| X-33 | wild-type | Mut$^+$ |
| GS115 | $his4$ | Mut$^+$, His$^+$ |
| KM71 | $his4$, $arg4$, $aox1::ARG4$ | Mut$^+$, His$^+$, Arg$^+$ |
| SMD1163 | $his4$, $pep4$, $prb1$ | Mut$^+$, His$^+$, Prot$^-$ (A$^-$, B$^+$, CarbY$^-$) |
| SMD1165 | $his4$, $prb1$ | Mut$^+$, His$^+$, Prot$^-$ (B$^+$) |
| SMD1168 | $his4$, $ura3$, $pep4::URA3$ | Mut$^+$, His$^+$, Prot$^-$ (A$^-$, B$^+$, CarbY$^-$) |
| PichiaPink® Strain 1 | $ade2$ | Mut$^+$, Ade$^+$ |
| PichiaPink® Strain 2 | $ade2$, $pep4$ | Mut$^+$, Ade$^+$, Prot$^-$ (A$^-$, B$^+$, CarbY$^-$) |
| PichiaPink® Strain 3 | $ade2$, $prb1$ | Mut$^+$, Ade$^+$, Prot$^-$ (B$^+$) |
| PichiaPink® Strain 4 | $ade2$, $pep4$, $prb1$ | Mut$^+$, Ade$^+$, Prot$^-$ (A$^-$, B$^+$, CarbY$^-$) |

a) NRRL, Northern Regional Research Laboratories, Peoria, IL. See text for the explanation of the different elements.
Table 3.2  *P. pastoris* expression vectors.

| Name                | Selection markers | Phenotype of transformants | Promoter | Secretion sequence | Added tags |
|---------------------|-------------------|----------------------------|----------|--------------------|------------|
| pAO815              | HIS4              | His<sup>+</sup>            | P<sub>AOX1</sub> | none               | none       |
| pPIC3.5K            | HIS4, Kan         | His<sup>+</sup>, G418<sup>a</sup> | P<sub>AOX1</sub> | none               | none       |
| pPIC9K              | HIS4              | His<sup>+</sup>, G418<sup>a</sup> | P<sub>AOX1</sub> | α factor           | none       |
| pPICZ a, B, C       | Ble               | Zeo<sup>R</sup>            | P<sub>AOX1</sub> | none               | -c-Myc/His<sub>6</sub> |
| pPICZα a, B, C      | Ble               | Zeo<sup>R</sup>            | P<sub>AOX1</sub> | α factor           | -c-Myc/His<sub>6</sub> |
| pPIC6 a, B, C       | Bsd               | Bla<sup>R</sup>            | P<sub>AOX1</sub> | none               | -c-Myc/His<sub>6</sub> |
| pHIL-D2             | HIS4              | His<sup>+</sup>            | P<sub>AOX1</sub> | none               | none       |
| pHIL-S2             | HIS4              | His<sup>+</sup>            | P<sub>AOX1</sub> | PHO1               | none       |
| pFLD                | Ble               | Zeo<sup>R</sup>            | P<sub>FLD1</sub> | none               | V5 epitope/His<sub>6</sub> |
| pFLDα               | Ble               | Zeo<sup>R</sup>            | P<sub>FLD1</sub> | α factor           | V5 epitope/His<sub>6</sub> |
| pGAPZ a, B, C       | Ble               | Zeo<sup>R</sup>            | P<sub>GAP</sub>  | none               | -c-Myc/His<sub>6</sub> |
| pGAPZα a, B, C      | Ble               | Zeo<sup>R</sup>            | P<sub>GAP</sub>  | α factor           | -c-Myc/His<sub>6</sub> |
| pPink-HC            | ADE2              | Ade<sup>+</sup>            | P<sub>AOX1</sub> | none               | none       |
| pPink-LC            | ADE2              | Ade<sup>+</sup>            | P<sub>AOX1</sub> | none               | none       |
| pPinkα-HC           | ADE2              | Ade<sup>+</sup>            | P<sub>AOX1</sub> | α factor           | none       |

*HIS4, *P. pastoris* auxotrophy marker, encodes a histidinol dehydrogenase; *Kan* gene, confers resistance to kanamycin (*Kan<sup>R</sup>, *E. coli*) and G418 (*G418<sup>R</sup>, *P. pastoris*); *Ble*, *Streptocellis hindustanus* ble gene, confers resistance to zeocin (*Zeo<sup>R</sup>); *Bsd* gene, confers resistance to blasticidin (*Bla<sup>R</sup>); *ADE2* gene, encodes a phosphoribosylaminimidazole carboxylase; P<sub>AOCX</sub>, promoter sequence of the alcohol oxidase-encoding AOX1 gene from *P. pastoris*; P<sub>FLD1</sub>, promoter sequence of the formaldehyde dehydrogenase-encoding FLD1 gene from *P. pastoris*; P<sub>GAP</sub>, promoter sequence of the glyceraldehyde-3-phosphate dehydrogenase-encoding GAP gene from *P. pastoris*; α factor, encodes the native *S. cerevisiae* α factor secretion signal; PHO1, encodes the native *P. pastoris* acid phosphatase secretion signal; V5 epitope, GKPIPNPLLGLDST peptide; c-Myc, C-terminal myc epitope, EQKLISEEDL.

With components required for *E. coli* amplification (classically one origin of replication and one antibiotic selection marker) and specific elements for heterologous gene expression in *P. pastoris*. These typically include selectable auxotrophy markers (*HIS4*, *ADE2* and/or antibiotic resistance bacterial genes (*bla*, *zeo*, and *kan*), a range of promoter and terminator sequences, a multiple cloning cassette, and supplementary signal sequences and other fusion sequences that can be added to improve the secretion and detection of the expressed proteins.

Among the panel of constitutive and inducible promoters that have been introduced in expression vectors (listed in [31]), P<sub>AOX1</sub> is by far the most widely used as it is the most strongly induced in the presence of methanol. Moreover, an original P<sub>AOX1</sub> synthetic promoter library was developed in a recent study that demonstrated enhanced P<sub>AOX1</sub> variants could reach higher expression levels of a tested recombinant Green Fluorescent Protein (GFP) [31].

A comprehensive set of vectors and strains is commercially available from Invitrogen, each of them being accessible either individually or included in expression kits.
3.4.3 Transformation and Clone Selection Strategies

As for many other yeasts, transformation of *P. pastoris* is rather straightforward. Several robust methods are available, either based on chemically competent (spheroplasts, PEG1000, LiCl) or electrocompetent cells, thus being accessible to a large majority of operators in standard labs. Moreover, these protocols are well described and can be easily found on numerous websites (convenient *Pichia* manuals can be downloaded from www.invitrogen.com).

Except for a limited set of autoreplicative plasmids that are not yet frequently employed [32–35], most of the transforming expression vectors are designed to be maintained as integrative elements in the genome of *P. pastoris*. This is generally achieved through recombination events between linearized sequences borne by the plasmids (typically *HIS4* or *P_AOX1*) and their homologous sequence counterparts present on the genome, leading to the targeted insertion of the expression vectors. Moreover, such plasmid insertions frequently occur in tandem in yeasts and thus lead to the multiple integration of the genes of interest with a correlated impact on their subsequent expression levels.

Alternatively, integration can be obtained by a gene replacement strategy. In this case, a double recombination event must be realized between the *AOX1* promoter and terminator sequences present on the transforming DNA (containing the gene of interest and a selection marker) and the corresponding homologous sequences present on *P. pastoris* genome. This double recombination event ends up with the replacement of the *AOX1* gene by the construct of interest.

The phenotype of the resulting transformants then depends not only on the selection marker present on the chosen vector (auxotrophy and/or antibiotic resistance), but also on the selected integration strategy (plasmid insertion versus gene replacement) that dictates their methanol utilization behavior. Indeed, while a plasmid insertion does not affect the methanol utilization ability of the transformed strain (Mut^+_, methanol utilization plus phenotype), the gene replacement of *AOX1* leads to a Mut^- (methanol utilization slow) phenotype. In several cases, these differences in methanol utilization have been reported as an important parameter to consider for enhancing the performance of recombinant protein expression [36].

3.4.4 Expression Conditions and Culturing Formats

Once transformants have been obtained, the next step usually consists in screening for the clones and conditions exhibiting the best expression levels of the recombinant protein. For expression strategies based on *P_AOX1*-dependent vectors, this is practically achieved by growing the cells in repressive media to an appropriate cell density and growth phase, before starting the production phase by transferring the cells to a methanol-containing induction media. For clones and expression condition screenings, small-scale culturing procedures most often rely on shaken baffled-flasks or on tubes of smaller volumes provided an appropriate aeration is
Successful Large-Scale Expression of Membrane Proteins Using P. pastoris

3.5.1 P. pastoris for Membrane Protein expression

The first use of P. pastoris as a host for the expression of an integral membrane protein was reported in 1995 when Helmut Reilander and his colleagues successfully expressed a member of the G-protein-coupled receptor (GPCR) family – the mouse serotonin receptor 5-HT$_{5A}$ [38]. Few additional membrane proteins were then assayed in the following years before the system became more and more popular in the 2000s: a thorough survey we conducted on the last decade of published results revealed 100 references encompassing more than 150 different membrane proteins expressed in P. pastoris (Table 3.3). In this list where only integral membrane proteins were considered (soluble domains of membrane proteins as well as membrane-anchored proteins were excluded), all classes of eukaryotic membrane proteins are equally represented. This includes monotopic receptors and enzymes, several aquaporins and ion channels, many members of the GPCR family, as well as large polytopic transporters bearing up to 17 putative transmembrane domains. This survey also highlights the great potential of P. pastoris for coexpression approaches, including studies on two membrane subunits of multimeric protein complexes, two interacting membrane protein partners, as well as a membrane protein and a cytosolic partner, in strategies where the coexpressed genes are either borne on a same vector or on two distinct vectors. Successful coexpression was actually recorded for the α and β subunits of Na/K-ATPase [39–42], and for α/β and phospholemman (a membrane modulator of the enzyme) [43]. Similarly, coexpression strategies were also reported for K$_{1,2}$, a membrane subunit, and K$_{β2}$, a cytosoluble partner, of the rat voltage-dependent K$^+$ channel [44–46].
| Protein name                                      | Organism       | kDa | Transmembrane domains | Strains          |
|--------------------------------------------------|----------------|-----|------------------------|------------------|
| Transports                                      |                |     |                        |                  |
| P-glycoprotein MDR3                              | mouse          | 140 | 12                     | GS115            |
| P-glycoproteins MDR3 (S430T, S1073T)             | mouse          | 140 | 12                     | GS115            |
| Multidrug resistance protein MRP1                | human          | 165 | 17                     | GS115, KM71      |
| Phosphate transporter MTP1                       | *Medicago truncatula* | 45  | ND                     | GS115            |
| Intestinal peptide transporter hPEPT1            | human          | 71  | ND                     | GS115            |
| P-glycoproteins MDR1, MDR3 (unglycosylated)      | human, mouse   | 140 | 12                     | GS115            |
| P-glycoprotein MDR1 (Cys-less)                   | human          | 140 | 12                     | GS115            |
| Antimalarial drug resistance protein Pfcrt (codon-optimized) | *Plasmodium falciparum* | 57  | 10                     | KM71, GS115      |
| P-glycoprotein MDR3                              | mouse          | 140 | 12                     | GS115            |
| Serotonin transporter rSERT                       | rat            | 50  | 12                     | GS115, SMD1168   |
| Breast cancer resistance protein BCRP            | human          | 62  | 6                      | KM71             |
| Sodium/glucose cotransporter hSGLT1              | human          | 55  | 9                      | GS115            |
| Copper transporter hCTR1                         | human          | 23  | 3                      | SMD1163          |
| Low-affinity cation transporter LCT1             | wheat          | ND  | ND                     | GS115            |
| Chloroquine resistance transporter PfCRT (codon-optimized) | *Plasmodium falciparum* | 45  | 10                     | KM71             |
| P-glycoprotein MDR3 (Cys-less)                   | mouse          | 140 | 12                     | GS115            |
| Multidrug resistance protein PfMDR1 (codon-optimized) | *Plasmodium falciparum* | 161 | 12                     | KM71, X-33       |
| 16 ABC transporters: ABCC3, ABC A1, A4, B1, C10, C11, C12, G5, G8, B7, B6, D1, E1, F1, G1, G4 | human         | 176 | 6, 12                  | KM71             |
| Glucose transporter NlHT1                        | *Nilaparvata lugens* | 40  | 12                     | X-33             |
### Table 3.3: Recombinant membrane proteins produced using the *P. pastoris* expression system.

| Protein name                  | Organism    | kDa | Transmembrane domains | Strains | Vector constructs (plasmid backbone) | Activity Description | Process      | Reference |
|-------------------------------|-------------|-----|------------------------|---------|-------------------------------------|----------------------|--------------|-----------|
| Transporters                  |             |     |                        |         |                                     |                      |              |           |
| P-glycoprotein MDR3           | mouse       | 140 | 12                     | GS115   | (pHIL-D2)-MDR3-His<sub>6</sub>-bio  | P: 4.3 μmol/min/mg, 0.35 mg/l | CESP         | [47]      |
| P-glycoproteins MDR3 (S430T, S1073T) | mouse     | 140 | 12                     | GS115   | (pHIL-D2)-mutMDR3-His<sub>6</sub>-bio | P: 3 μmol/min/mg, 0.7 mg/l | CESP         | [48]      |
| Multidrug resistance protein MRP1 | human      | 165 | 17                     | GS115, KM71 | (pHIL-D2)-MRP1-HA-His<sub>6</sub> | ligand binding assay | CE           | [49]      |
| Phosphate transporter MtPT1   | *Medicago truncatula* | 45  | ND                     | GS115   | (pPIC3K)-MtPT1 | functional complementation | CE           |           |
| Intestinal peptide transporter hPEPT1 | human | 71  | ND                     | GS115   | (pGAPZB)-hPEPT1-c-Myc-His<sub>6</sub> | transport assay, E: 64 pmol/mg | CE           | [51]      |
| P-glycoproteins MDR1, MDR3 (unglycosylated) | human, mouse | 140 | 12                     | GS115   | (pHIL-D2)-QQQ-MDR1-His<sub>10</sub>, (pHIL-D2)-QQQ-MDR3-His<sub>6</sub> | ATPase activity, P: 1.2–3.8 U/mg, 0.75–1.25 mg/l | CESP         | [52]      |
| P-glycoprotein MDR1 (Cys-less) | human       | 140 | 12                     | GS115   | (pHIL-D2)-MDR1-His<sub>10</sub> | ATPase activity | CESP         | [53]      |
| Antimalarial drug resistance protein Pfcrt (codon-optimized) | *Plasmodium falciparum* | 57  | 10                     | KM71, GS115 | (pPIC3.5)-Pfcrt-bio | ATPase activity | CESP         | [54]      |
| Breast cancer resistance protein BCRP | human      | 62  | 6                      | KM71    | (pHIL)-BCRP-His<sub>10</sub> | ATPase activity, ligand binding assay, E: 80 nmol/min/mg | CE           | [55]      |
| Sodium/glucose cotransporter hSGLT1 | human      | 55  | 9                      | GS115   | (pPICZB)-hSGLT1-FLAG-His<sub>6</sub> | functional transport, E: 273 nmol/min/mg, P: 3 mg/l | CESPC         | [57]      |
| Copper transporter hCTR1      | human       | 23  | 3                      | SMD1163 | (pPIC3.5K)-HA-hCTR1N15Q-His<sub>6</sub> | functional complementation | CESP         | [58]      |
| Low-affinity cation transporter LCT1 | *wheat*   | ND  | ND                     | GS115   | (pPIC3.5K)-LCT1 | transport activity, E: 14 pmol/10<sup>6</sup> cells/10 min | CE           | [59]      |
| Chloroquine resistance transporter PfCRT (codon-optimized) | *Plasmodium falciparum* | 45  | 10                     | KM71    | (pPICZA)-CRT-His<sub>10</sub> | transport activity, P: 487 pmol/mg/min | CESP         | [60]      |
| P-glycoprotein MDR3 (Cys-less) | mouse       | 140 | 12                     | GS115   | (pHIL-D2)-MDR3-His<sub>10</sub> | ATPase activity | CESP         | [61]      |
| Multidrug resistance protein PfMDR1 (codon-optimized) | *Plasmodium falciparum* | 161 | 12                     | KM71, X-33 | (pPICZc/pPIC3.5)-PfMDR1-His<sub>6</sub>-bio | ATPase activity, P: 63 U/mg/minute | CESP         | [62]      |
| 16 ABC transporters: ABCC3, ABC A1, A4, B1, C10, C11, C12, G5, G8, B7, B6, D1, E1, F1, G1, G4 | human      | 176 | 6, 12                  | KM71    | (pSGP18)-ABC-CBP-His<sub>6</sub> | ATPase activity, P: 82 nmol/min/mg, P: 35 mg/g cells | CESP (ABCC3) | [63]      |
| Glucose transporter NlHT1     | *Nilaparvata lugens* | 40  | 12                     | X-33    | (pPICZB)-NlHT1-c-Myc-His<sub>6</sub> | transport activity | CE           | [64]      |

(Continued)
Table 3.3 (Continued)

| Protein name                                      | Organism          | kDa | Transmembrane domains | Strains    |
|---------------------------------------------------|-------------------|-----|------------------------|------------|
| Vesicular glutamate transporter VGLUT1            | rat               | 61  | 12                     | X-33       |
| Glucose transporters GLUT1 and GLUT4              | human, rat        | 42, 46 | 12                     | X-33       |
| P-glycoprotein Pgp                                | mouse             | ND  | 12                     | GS115      |
| Formate-nitrite transporter AnNitA                | Aspergillus nidulans | 31  | 6, 8                   | GS115      |
| Water channel proteins                            |                   |     |                        |            |
| Aquaporin PM28A                                   | spinach           | 32  | 6                      | X-33       |
| Aquaporin SoPIP2;1                                | spinach           | 32  | 6                      | ND         |
| Aquaporin PvTIP3;1                                | plant             | 25  | 6                      | KM71       |
| Aquaporin SoPIP2.1                                | spinach           | 32  | 6                      | X-33       |
| Aquaporin AQP6                                    | rat               | 29  | 6                      | X-33, GS115, KM71 |
| Aquaporin hAQP1                                   | human             | 35  | 6                      | X-33       |
| Aquaglyceropin PfAQP (codon-optimized)            | Plasmodium falciparum | 30  | 6                      | X-33       |
| Aquaporin HsAQP5                                  | human             | ND  | 6                      | ND         |
| Aquaporin AtPIP2.1                                | Arabidopsis thaliana | 55  | 6                      | X-33       |
| Aquaporins TgPIP2;1, TgPIP2;2                     | Tulipa gesneriana | 31  | 6                      | KM71       |
| Aquaporin Aqy1                                   | Pichia pastoris   | ND  | 6                      | GS115 aqy1 |
| Aquaporin hAQP4                                   | human             | ND  | 6                      | X-33       |
| 13 Aquaporins (hAQP0 to hAQP12)                    | human             | 30  | 6                      | X-33       |
| Ion channel proteins                              |                   |     |                        |            |
| Voltage-sensitive K⁺ channel K₁,₂/β₂ (coexpression)| rat               | 58/40 | 6                     | SMD1163    |
| Voltage-dependent K⁺ channel K₁,₂/β₂ (coexpression)| rat               | ND  | 6                      | SMD1163    |
| Calcium-activated K⁺ channel SK2                  | mammalian         | 64  | 6                      | SMD1163    |
| Chimeric K⁺ channel K₁,₂/ K₂,1/β₂,1 (coexpression)| rat               | ND  | 6                      | SMD1163    |
| Inward-rectifier K⁺ channel K₂,2                  | chicken           | ND  | 6                      | SMD1163    |
### 3.5 Successful Large-Scale Expression of Membrane Proteins Using *P. pastoris*

| Vector constructs (plasmid backbone) | Activity | Process | Reference |
|-------------------------------------|----------|---------|-----------|
| (pGAPZB)-c-Myc-His-VG1UT1           | P: 1 mg/l | CESP    | [65]      |
| (pPICZB)-GLUT-His<sub>s</sub>       | transport activity, P: 13.1 mg/g cells | CESP   | [66]      |
| (pHIL-D2)-QQQ-Pgp-His<sub>s</sub>   | ATPase activity | CESP (3.8 Å) | [67] |
| (pPICZA)-His<sub>g</sub>-AnNitA     | ND       | CESP    | [68]      |
| (pPICZB)-PM28A-c-Myc-His<sub>s</sub> | P: 25 mg/l | CESP    | [69]      |
| (ND)-SoPIP2:1                       | water channel activity | CESPCS (5 Å) | [70] |
| (pPICZ)-PrTIP3:1-gly3-His<sub>s</sub>| water channel activity | CE     | [71]      |
| (pPICZB)-SoPIP2.1 ± His<sub>s</sub> | P: 25 mg/l | CESPCS (2.1, 3.9 Å) | [72] |
| (pPICHO)l/pPICZ)-AQP6-His<sub>s</sub>| E: 7 pmol/mg | CE     | [73]      |
| (pPICZB)-hAQP1-c-Myc-His<sub>s</sub>| water channel activity, P: 90 mg/l | CESP    | [74]      |
| (pPICZB)-PfAQP-c-Myc-His<sub>s</sub>| P: 18 mg/l | CESP    | [75]      |
| (pPICZB)-HsAQP5                      | water channel activity | CESPCS (2 Å) | [76] |
| (pPICZB)-WT/mutAtPIP2:1             | water channel activity, P: 65 μg/l | CESP | [77]      |
| (pPICZB)-TgPIP2-gly3-His<sub>s</sub>| water channel activity | CE | [78]      |
| (pPICZaB)-ΔN36Aqp1-His<sub>s</sub> | water channel activity | CESPCS (1.5 Å) | [79] |
| (pPICZ)-His<sub>g</sub>-FLAG-hAQP4  | water channel activity | CESPCS (1.8 Å) | [80] |
| (pPICZB)-hAQP-His<sub>s</sub>       | water channel activity | CE     | [81]      |
| (pPIC3.5K)-His<sub>s</sub>-K<sub>1.2</sub>: | E: 98 pmol/mg, P: 26 mg, 3.3 nmol/mg | CESPCS (2.1 nm) | [44] |
| (pPICZC)-streplf1-K<sub>β</sub>     | P: 10 mg/ml | CESPCS (2.9 Å) | [45] |
| (pPICZC)-His<sub>s</sub>-K<sub>1.2</sub>-<sub>β</sub>2 | E: 0.1 pmol/mg | CES | [82] |
| (pPIC3.5K)-streplf1-His-SK2         | channel activation | CESPCS (2.4 Å) | [46] |
| (pPICZC)-His<sub>s</sub>-K<sub>1.2</sub>/K<sub>2.1</sub>-<sub>β</sub>2.1 | channel activation, P: 8 mg/ml | CESPCS (3.1 Å) | [83] |

(Continued)
### Table 3.3 (Continued)

| Protein name                        | Organism | kDa | Transmembrane domains | Strains               |
|-------------------------------------|----------|-----|------------------------|-----------------------|
| GPCRs                               |          |     |                        |                       |
| Endothelin receptor B ETB           | human    | 55  | 7                      | SMD1163               |
| Endothelin receptor B ETB           | human    | 55  | 7                      | SMD1163               |
| Cannabinoid receptor CB2            | human    | 51  | 7                      | X-33                  |
| Mu-opioid receptor HuMOR            | human    | 74  | 7                      | GS115, SMD1163, SMD1168, X-33 |
| Dopamine receptor D_{2S}            | human    | 40  | 7                      | SMD1163               |
| Dopamine receptor D_{2S}            | human    | 51  | 7                      | SMD1163               |
| Receptor smoothened hSmo             | human    | 80  | 7                      | GS115                 |
| Cannabinoid receptor CB1            | human    | 75  | 7                      | X-33                  |
| Mu-opioid receptor HuMOR            | human    | 45/66| 7                      | SMD1163               |
| 20 GPCRs: ADA1B, ADA2B, ACM1, ACM2, HRH2, OPRK, SHT_{1D}, SHT_{1B}, SHT_{1A}, DRD2, NK1R, NK2R, NK3R, NPY1R, AA_{2A}R | human, pig, mouse, rat | ND | 7                      | SMD1163               |
| Adenosine A_{2A} receptor hA_{2A}R  | human    | 34  | 7                      | SMD1163               |
| β_{2}-Adrenergic receptor β_{2}AR   | human    | 45  | 7                      | SMD1168               |
| (codon-optimized)                   |          |     |                        |                       |
| 100 GPCRs                           | human, pig, mouse, rat, bovine, yeast | 36–126 | 7                      | SMD1163               |
| EDG-1 receptors                     | human    | 69  | 7                      | SMD1168               |
| Bradykinin B2 receptor B2R          | human    | 68  | 7                      | GS115                 |
| Bradykinin B2 receptor B2R          | human    | 55  | 7                      | SMD1163               |
| Protein name                  | Organism  | kDa | Transmembrane domains | Strains          | Vector constructs (plasmid backbone) | Activity          | Process | Reference |
|------------------------------|-----------|-----|-----------------------|------------------|-------------------------------------|-------------------|---------|-----------|
| GPCRs: ADA1B, ADA2B, ACM1, ACM2, HRH2, OPRK, 5HT1D, 5HT1B, 5HT1A, DRD2, NK1R, NK2R, NK3R, NPY1R, AA2A R | human, pig, mouse, rat | ND | 7 | SMD1163 | (pPIC9K)-AF-FLAG-His10-GPCR-bio | E: 0.3–165 pmol/mg | CE | [97] |
| Dopamine receptor D2S | human | 40 | 7 | SMD1163 | (pPIC9K)-AF-FLAG-±His6-D2S | P: 10 pmol/mg | CESP | [89] |
| Receptor smoothened hSmo | human | 80 | 7 | GS115 | (pAO815)-hSmo-CBD-strep-HA-His6 | ND | CESP | [91] |
| Cannabinoid receptor CB1 | human | 75 | 7 | X-33 | (pPICZa)-AF-FLAG-CB1-c-Myc-His6 | E: 3.6 pmol/mg | CESP | [92] |
| Mu-opioid receptor HuMOR | human | 45/66 | 7 | SMD1163 | (pPICZ)±GFP-HuMOR-c-Myc-His6 | E: 0.45 pmol/mg | CESP | [93] |
| 20 GPCRs: ADA1B, ADA2B, ACM1, ACM2, HRH2, OPRK, 5HT1D, 5HT1B, 5HT1A, DRD2, NK1R, NK2R, NK3R, NPY1R, AA2A R | human, pig, mouse, rat | 36–126 | 7 | SMD1163 | (pPIC9K)-AF-FLAG-His10-GPCR-bio | E: 0.1–180 pmol/mg | CE | [97] |
| Adenosine A2A receptor hA2AR | human | 34 | 7 | SMD1163 | (pPICZaA)-AF-FLAG-His10-hA2AR | P: 18 nmol/mg | CESP | [95] |
| β2-Adrenergic receptor β2AR | human | 45 | 7 | SMD1168 | (pPIC9K)-AF-His6-β2AR | P: 11 nmol/mg | CESP | [96] |
| Bradykinin B2 receptor B2R | human | 68 | 7 | GS115 | (pPIC9K)-AF-B2R-GFP | E: 8.2 pmol/mg | CES | [98] |
| Bradykinin B2 receptor B2R | human | 55 | 7 | SMD1163 | (pPIC9K)-AF-FLAG-ΔPETB-bio/B2R | E: 3.5 pmol/mg | CE | [100] |

(Continued)
### Table 3.3 (Continued)

| Protein name                        | Organism    | kDa  | Transmembrane domains | Strains  |
|-------------------------------------|-------------|------|-----------------------|----------|
| Neuromedin U receptor NmU2R         | human       | 60   | 7                     | SMD1163  |
| Cannabinoid receptor CB2            | human       | 42   | 7                     | X-33     |
| 12 GPCRs: CNR2, NK1R, NK3R, ADA1B, ADA2B, ADA2C, D2DR, OPRK, OPRD, P2RY1, HRH1, PAR1 | human, rat  | 40–50 | 7                     | SMD1163  |
| Mu-opioid receptor (ΔN64) ΔN64-HuMOR | human       | 38   | 7                     | SMD1163  |
| β-Adrenergic receptors β1AR, β2AR, β3AR | human       | 66   | 7                     | SMD1163  |
| 25 nonglycosylated GPCRs: CNR1, AGTR2, HTR1B, ADORA2A, DRD1, DRD2, DRD4, DRD5, OPRK, CHRM2, PTGER1, PTGER2, PTGER3, PTGER4, TBXRA2, TACR1, TACR2, TACR3, NTSR1, ADRB2, HRH4 | human, mouse | ND   | 7                     | SMD1163  |
| Leukotriene B, receptor BLT1        | guinea pig  | 100  | 7                     | GS115    |
| Enzymes                             |             |      |                       |          |
| 11β-OH steroid dehydrogenase 11β-HSD1 | human       | 29   | ND                    | GS115    |
| Monoamine oxidase B MAOB           | human       | 60   | 1                     | KM71, GS115 |
| Monoamine oxidase B MAOB           | human       | 60   | 1                     | KM71, GS115 |
| Monoamine oxidase A MAOA           | human       | 60   | 1                     | KM71     |
| 11β-OH steroid dehydrogenases 11β-HSD1 | human, rat  | 31   | ND                    | GS115, X-33 |
| Isatin-bound monoamine oxidase B MAOB | human       | 60   | 1                     | KM71, GS115 |
| Na/K-ATPase (α, β) (coexpression)  | pig         | 112/47 | 10, 1                | SMD1165  |
| Oxidosqualene cyclase hOSC          | human       | 80   | 1                     | GS115    |
| Na/K-ATPase (α, β) (coexpression)  | pig         | ND   | 10, 1                 | SMD1165  |
| Vector constructs (plasmid backbone) | Activity | Process | Reference |
|-------------------------------------|----------|---------|-----------|
| (pPIC9K)-AF-FLAG-His<sub>10</sub>-NmU2R-bio | E: 6 pmol/mg | CE | [21] |
| (pPICZa)-AF-FLAG-CB2-c-Myc-His<sub>6</sub> (pPICZa)-AF-FLAG-CB2-His<sub>6</sub>/His<sub>6</sub> | ligand binding assay | CESP | [101] |
| (pPIC9K)-AF-FLAG-His<sub>10</sub>-GPCR-bio | ligand binding assay, P: up to 0.9 mg/l | CESP | [102] |
| (pPICZB) ± AF ± GFP-ΔN64-HuMOR-c-Myc-His<sub>6</sub> | P: 5 mg/l | CESP | [103] |
| (pPICZ)-AF-GFP-βAR-c-Myc-His<sub>6</sub> | ND | CESP | [104] |
| (pPIC9K)-AF-FLAG-GPCR-His<sub>10</sub> | E: 0–75.4 pmol/mg | CE | [22] |
| (pPIC3.5K)-AF-FLAG-BLT1 | E: 50 pmol/mg, P: 0.4 mg/l | CESP | [105] |
| (pPIC3.5K)-11β-HSD1 | enzyme activity | CE | [106] |
| (pPIC3.5K)-MAOB | oxidase activity P: 100 mg/l | CESP | [107] |
| (pPIC3.5K)-MAOB | ND | CESP | [108] |
| (pPIC3.5K)-MAOA | P: 115 mg/l | CESP | [109] |
| (pPIC3.5K)/pPICZB)-His<sub>10</sub>-11β-HSD1 | dehydrogenase activity, S: 713 pmol/min/mg | CESP | [110] |
| (pPIC3.5K)-MAOB | ND | CESP | [111] |
| (pHIL-D2)-α<sub>1</sub>/β<sub>1</sub> | E: 30–50 pmol/mg | CE | [39] |
| (pPICZB)-hOSC-c-Myc-His<sub>6</sub> | IC<sub>50</sub> values, P: 105 mg/l | CESP | [112] |
| (pHIL-D2)-α<sub>1</sub>/His<sub>10</sub>β | E: 30 pmol/mg, 1 g/31 | CESP | [40] |

(Continued)
| Protein name                                      | Organism     | kDa  | Transmembrane domains | Strains   |
|--------------------------------------------------|--------------|------|-----------------------|-----------|
| Clorgyline-bound monoamine oxidase A MAOA        | human        | 60   | 1                     | KM71      |
| Cytochrome P450 2D6 monoxygenase CYP2D6/ NADPH P450 oxidoreductase CPR (coexpression) | human        | 56/77| 1, 1                  | X-33      |
| Adrenal cytochrome b561 Cytb561                  | bovine       | 28   | 5, 6                  | GS115     |
| Cytochrome P450 PcCYP1f                           | *Phanerochaete chrysosporium* | 60   | ND                    | KM71      |
| Phospholemman (PLM)/ Na/K-ATPase (*α, β*)         | human PLM, pig (*α, β*) | ND   | 1, 1, 1               | SMD1165   |
| Na/K-ATPase (*α, β*) (coexpression)               | human, pig   | ND   | 10, 1                 | SMD1165   |
| Cytochrome P450 17α-hydroxylase CYP17             | human        | 54   | ND                    | GS115     |
| Apo and GSH-complexed leukotriene C4 synthase LTC4S | human        | ND   | 4                     | KM71H     |
| Na/K-ATPase (*α, β*) (coexpression)               | pig          | 110/44| 10, 1                 | GS115,    |
| Monoamine oxidase B MAOB                         | rat          | 60   | 1                     | KM71      |
| Leukotriene C4 synthase LTC4S                    | rat          | 18   | 4                     | KM71      |
| Monoamine oxidase A MAOA                         | rat          | 60   | 1                     | KM71      |
| Monoamine oxidase MAO                            | zebrafish    | 60   | 1                     | KM71      |
| Other membrane proteins                          |              |      |                       |           |
| Thromboplastin, tissue factor TF                  | rabbit       | 31   | 1                     | GS115     |
| Immunotoxin Cyt2Aa1 (codon-optimized)            | *Bacillus thuringiensis* | 60   | 1?                    | KM71      |
| Lectin-like oxLDL receptor 1 hLOX-1               | human        | 43   | 1?                    | GS115     |

Table 3.3 (Continued)
## 3.5 Successful Large-Scale Expression of Membrane Proteins Using P. pastoris

| Vector constructs (plasmid backbone) | Activity | Process | Reference |
|-------------------------------------|----------|---------|-----------|
| (pPIC3.5K)-MAOA                     | P: 115 mg/l | CESPCS (3 Å) | [113] |
| (pPIC3.5K)-MAOB                     | oxidase activity, P: 100 mg/0.5 l | CESP | [119] |
| (pPIC3.5K)-LTC4S                    | P: 1 mg/l, 49 µmol/mg/min | CESPC | [120] |
| (pPIC9K)-zMAO                       | E: 700 U/l, P: 200 mg/l | CESP | [121] |
| (pPIC3.5K)-zMAO                     | P: 200 mg/l, 300 U/l | CESP | [122] |
| (pII-D26)-PHO1-TF-His6              | P: 0.1 mg/g cells | CESP | [123] |
| (pPICZB)-CsFvC6.5-synCyt2Aa1-c-Myc-His6 | cytotoxic activity, P: 10 mg/l | CESP | [124] |
| (pPIC9K)-AF-hLOX-1-His6             | ND | CESP | [125] |

(Continued)
Moreover, this list not only reports on expression evaluations in *P. pastoris* but also covers solubilization, purification, crystallization, and structural studies of membrane proteins produced with this system. Remarkably, high-resolution structures for a dozen of them were thus obtained (see CEPSCS-labeled references in Table 3.3), which represents about one-third of the recombinantly produced eukaryotic membrane proteins for which a three-dimensional structure is available as of January 2010 (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Overall, these records highlight *P. pastoris* as one of the most performant heterologous expression system for the structural studies of eukaryotic membrane proteins.

### 3.5.2 Common Trends for an Efficient Expression of Membrane Proteins in *P. pastoris*

The basic experimental data recorded in Table 3.3 are intended to give some general directions to help the reader in the choice of an adapted procedure to start with for his/her favorite membrane protein to be expressed in *P. pastoris*.
3.5 Successful Large-Scale Expression of Membrane Proteins Using P. pastoris

| Vector constructs (plasmid backbone) | Activity | Process | Reference |
|-------------------------------------|----------|---------|-----------|
| (pPICZB/pPICZa) ± AF-MP-c-Myc-His6 | ND       | CESP    | [126]     |
| (pPICZB)-hCD81-His6                 | P: 1.75 mg/l | CESP    | [127]     |
| (pPICZA)-His6-PMP22                 | P: 90 mg/41 | CESP    | [128]     |
| (pPICZA)-p/RDS-c-Myc-His6           | P: 0.3 mg/l | CESP    | [129]     |
| (pPICZaA)-AF-HER-2/neu-c-Myc-His6   | ND       | CE      | [130]     |

Regarding the plasmidic constructs to select, nearly all kinds of available expression vectors have been assayed, most exclusively based on the $P_{\text{AOX1}}$ inducible promoter. To our knowledge, only three noticeable exceptions are reported on the use of vectors bearing the constitutive promoter $P_{\text{GAP}}$ for the expression evaluation of membrane proteins, including the human intestinal peptide transporter hPEPT1 [51], the human phospholemman [43], and the rat vesicular glutamate transporter 1 VGLUT1 [65]. In all cases the expression levels were rather significant; however, no comparison was conducted on the benefit of such a constitutive expression over an inducible system.

Overall, no real tendency emerges in the choice of a given vector for a given type of membrane protein, except in the case of water channel proteins where pPICZ constructs were always privileged. Similarly, whereas a secretion sequence is quite systematically added upstream of GPCR open reading frames (ORFs) for an enhanced expression of functional proteins, there is no apparent rule for all the other reported membrane proteins whatever their topology and orientation in the membrane. In the case of the six-transmembrane domain aquaporins, for instance, where N- and C-termini are intracellularly located, protein expression has been evaluated with or without a fused secretion sequence and both situations proved efficient enough to obtain high-resolution structures of the produced protein ([79] versus [72, 76, 80]).
As for several other expression systems, a large panel of tag sequences are frequently inserted to improve the downstream detection and purification steps, ranging from hexa- or decahistidine (the most widely employed tags), c-Myc, FLAG, HA and StreptII epitopes, to larger peptidic domains such as a biotinylation domain (bio), a calmodulin binding domain (CBD), or the fluorescent protein GFP. In addition, protease cleavage sequences are sometimes included in the constructs such as the Factor Xa or tobacco etch virus (TEV) sequences in order to eliminate the fused tags after or during the purification step.

Nearly all commercially available strains have been used to express membrane proteins. While the criteria used for the choice of a given strain are generally not documented, few studies reported on the membrane protein-dependent differential behavior of strains – a phenomenon that is commonly observed. SMD1163, KM71, and X-33 indeed appeared more performant than GS115 for the expression of a GPCR [38], an ATP-binding cassette (ABC) transporter [49], and a tetraspanin [127], respectively, whereas GS115 performed better than SMD1168 for the recombinant expression of a serotonin transporter [20]. Alternatively, no real variation of the expression level could be observed when a GPCR, the μ-opioid receptor, was evaluated in the X-33, GS115, SMD1163, and SMD1168 strains [87].

In most of the reported studies, functional expression levels of membrane proteins are assessed through both specific immunodetection tests and activity assays. In the situation where these parameters can be compared, as in the particular case of GPCRs, the outcome highlights a very fluctuating performance of the system that not only depends on the expressed membrane protein, but also on the experimental conditions assayed. Therefore, optimization of the expression conditions is often very helpful for the recovery of higher amounts of functional recombinant membrane proteins. This is the issue of the next section that aims at illustrating how expression levels can be enhanced with GPCRs as model membrane proteins.

### 3.6 Guidelines for Optimizing Membrane Protein Expression in *P. pastoris* Using GPCRs as Models

From the seminal work of Weiss *et al.* [38], more than 30 original articles focusing on GPCR expression in *P. pastoris* have been published so far. This wealth of quantitative and qualitative information relative to hundreds of different receptors from the same membrane protein family represents an ideal source of data to exemplify the different directions that can be undertaken to enhance the expression levels of membrane proteins. Different experimental adjustments conducted in several of these studies indeed proved highly beneficial. They can be divided into two main categories that are detailed below: those allowing us to design and to select for the most performing clones, and those implemented at the level of growth and induction. In addition, some considerations on optimizing yeast cell lysis are also briefly discussed.
3.6.1 Design and Selection of Enhanced Expression Clones

As a general rule in heterologous expression studies, optimizing the coding sequence of the gene to be expressed is often very helpful. For instance, fitting the gene sequence to the codon usage of the host organism has generally shown a beneficial impact on expression levels (reviewed in [131]). In the case of GPCRs expressed in \textit{P. pastoris}, a codon-optimized human \(\beta_2\)-adrenergic receptor (\(\beta_2\)AR) exhibited an activity of 6 pmol/mg in total membrane preparations [96] – a figure to be compared with the 24 pmol/mg functional receptors that were obtained for a nonoptimized human \(\beta_2\)AR expressed in a quite similar context [19]. This codon-optimization engineering of a GPCR thus did not appear really profitable, albeit this single reported approach may probably not be representative and more data are needed. Similarly, with the double goal of improving expression levels and receptor homogeneity, direct mutagenesis of potential N-glycosylated residues was evaluated on several GPCRs and revealed a rather average outcome: whereas the receptor homogeneity was generally enhanced, the specific activity of the receptors was lowered in most cases [22, 95], only few of them being improved [22, 84] or remaining unchanged [90]. Larger sequence modifications were also reported, showing important beneficial effects for a C-terminally truncated adenosine A\(_2\)A receptor [132] and for a 47-amino-acid deletion of an internal loop for an acetylcholine muscarinic receptor [22], but with no real impact on expression levels in the case of a N-terminal deletion of a \(\mu\)-opioid receptor [103, 133].

Introducing additional fusion sequences may sometimes reveal a fruitful way to increase expression of GPCRs. Several studies notably compared the benefit of secretion signals added upstream the gene of interest, showing a very substantial effect of the signal sequence of the \(\alpha\)-factor from \textit{S. cerevisiae} on the expression levels of a serotonergic [38], an opioid [103, 133], and a dopaminergic [90] receptor. This signal sequence is since systematically inserted for GPCR expression.

Addition of short tag sequences including the hexa- or decahistidine, FLAG or the c-Myc regularly proved very useful for the downstream detection and/or purification procedures of GPCRs, but did not result in significant changes in their expression levels. Similarly, GFP fused to several GPCRs either C-terminally [85, 98, 99] or N-terminally [103, 104] did not markedly modify their expression profile. Instead, the GFP fusion appeared a useful multipurpose tool for (i) the selection of overexpressing clones, (ii) the determination of total recombinant protein expression, (iii) the evaluation of solubilization and purification conditions, and (iv) the subcellular localization of the receptors.

Interestingly, a significantly increased production level was observed when the biotinylation domain of the transcarboxylase from \textit{Propionibacterium shermanii} was fused to the C-terminus of several receptors. For instance, for 5-HT\(_{5A}\) [19], human ET\(_B\) endothelin receptor (ETBR) [85], DRD2 [90], and \(\beta_2\)AR (C. Reinhart, personal communication) the number of active receptors per cell was more than doubled. In addition, the absence of this sequence from the GPCR constructs used in the study of Yurugi-Kobayashi \textit{et al.} [22] probably participated to the lower expression
levels observed for several of them when compared to the same receptors bearing this biotinylation domain [97]. Addition of this domain likely stabilized the recombinant receptor either by protecting the receptor from direct degradation or maintaining folding fidelity to avoid the unfolded protein response.

Regarding the choice of the cellular host to use, several studies compared the benefit of one strain over the others. For instance, the receptors 5-HT\textsubscript{5A} and ETA were respectively expressed at higher levels in the protease-deficient strains SMD1163 [38] and SMD1168 [134] than in the GS115 strain (see Table 3.1 for the description of the strains). Similarly, the strain SMD1168 appeared a most appropriate host for the expression of a CB2 receptor when compared to the strain X-33 [101]. In several other cases, however, no significant differences were observed, and strains from the SMD series were mainly retained because of their protease-deficient properties and their inherent lower impact on protein degradation during the downstream preparative steps.

In addition, since multicopy integration events occur with a relatively high frequency in \textit{P. pastoris} transformants, gene dosage is also an important issue that directly impacts expression levels. For instance, a panel of clones resistant to increasing concentrations of zeocin [88] and G418 (geneticine) [19, 90] was selected for its representative content of integrated GPCR gene copy number. These studies and others show that the levels of active receptors increased correlatively with the number of integrated copies up to a plateau after which additional copies had no effect. Most importantly, from our observations (unpublished data), clones bearing the highest antibiotic resistance levels (i.e., the highest copy number of GPCRs genes) were often those presenting the highest amounts of immunodetected receptors, whereas ligand-binding activities were not improved. Such clones are thus generally not desired as they display a large proportion of nonfunctional receptors. Moreover, these observations strongly suggest that the bottleneck for the production of functional receptors lies in folding and/or post-translational processing rather than in the transcription and translation steps. As a consequence, clone selection procedures have to rely both on a representative phenotypic screening followed by an appropriate evaluation of the receptor quantity and activity.

### 3.6.2 Optimization of the Expression Conditions

Once the most performant clones have been selected, further improvements can be implemented by appropriately adjusting some of the experimental parameters that influence the host cell physiology, and hence its performance for heterologous gene expression, correct protein folding, and proper trafficking. These external factors include culture format and procedures, temperature and time of induction, cell densities, formulation of growth media, or supplementation with stabilizing compounds or chemical chaperones.

As a first step, evaluating the production time course of a GPCR is often very useful as the outcome may vary importantly from one receptor to another. For
instance, 10h was determined as the optimal induction time for different constructs expressing a µ-opioid receptor [87], whereas the highest expression levels were obtained in the range of 18–24 h postinduction for a majority of other receptors and up to 60h for an engineered ACM2 muscarinic receptor [22]. Similarly, while the induction phase in methanol-containing media is usually performed using cell densities of about $5 \times 10^7$ cells/ml (1 OD$_{600}$/ml), we observed that this parameter was differently affecting the expression level of GPCRs, higher cell densities (up to 10 OD$_{600}$/ml) being actually more appropriate for several of them (unpublished data).

Formulation of the induction media is also an important issue. Adjustments in the composition of buffered media, pH values, and methanol concentration usually did not bring major benefits in GPCR expression, and a typical induction is generally performed at pH 5–7 in buffered complex media containing 0.5% methanol. Much more substantial improvements, however, can be gained by supplementing these media with some small compounds that are believed to facilitate the folding and processing of the recombinant proteins. Among these molecules, dimethyl sulfoxide (DMSO) added in the induction medium remarkably increased the production yield of 16 out of 20 tested receptors up to 6-fold relative to standard conditions [94]. Similar effects were observed in other studies evaluating GPCR expression not only in the P. pastoris system [21, 100], but also using mammalian [21] or insect [135] cells hosts. The precise role of DMSO here is not clear, but it has been shown to dramatically alter the membrane properties of several organisms by increasing their permeability [136], thus possibly influencing the processes of membrane protein translocation. DMSO is also thought to act as a stabilizer of folding intermediates and has been already qualified a chemical chaperone [137]. In a comparable fashion, adding ligands specific to a given GPCR proved highly beneficial for a large majority of tested receptors [19, 90, 94]. In the case of a histaminergic H$_2$ receptor, the expression level was improved up to 7-fold in the presence of an antagonist compound, cimetidine, in the induction medium [94]. Such small molecules are considered pharmacological chaperones as they have been shown to selectivity promote the proper folding and trafficking of the targeted GPCR [137], therefore limiting the recurrent complications related to misfolding/aggregation and misfolding/degredation pathways.

In addition, it was shown in several studies that lowering temperature during expression to a typical range of 18–24°C was optimal for various receptors, as measured by ligand binding [22, 87, 90, 94, 95]. Possible explanations for the temperature effect include slowing down protein production and not overloading the translocation machinery, protein processing, or intracellular trafficking. Lowering temperature has also been shown to reduce proteolytic activities and upregulate cold shock proteins such as chaperones.

As illustrated in a work we conducted on a selection of 20 GPCRs [94], adjusting these different parameters at the culturing level always turned out beneficial and every tested clones revealed higher ligand binding values ($B_{\text{max}}$) compared with the standard condition. Strikingly, eight out of these 20 receptors revealed high $B_{\text{max}}$ values (above 20 pmol/mg) after optimization. In addition, we and others also
found that the amount of functional receptor (in terms of ligand binding) did not scale with the total amount of receptors evaluated either by immunodetection [94] or by fluorescence [88] measurements. Most importantly, the total amount of receptors was not changed after optimization while the total number of binding sites ($B_{\text{max}}$) was increased from 1.3- to more than 8-fold. These data are all in agreement with the concept that GPCRs are expressed in *P. pastoris* under a functional/nonfunctional equilibrium that can be modulated with the expression conditions that are used.

### 3.6.3 Yeast Cell Lysis

As a common trait of budding yeasts, *P. pastoris* possesses a thick protective cell wall that requires the use of aggressive disruption methods for the recovery and preparation of the membrane protein-containing fractions. The choice of a cell lysis method suited to *P. pastoris* cultured in various volumes and formats is therefore nontrivial, albeit very few studies report on this important issue [27]. We here indicate a selection of techniques and apparatus references that we and others found the most adapted to this key step.

Shearing-based methods involving microbeads are mostly preferred as they are very efficient, compatible with a broad range of sample volumes, and directly accessible to most of the standard-equipped labs. In the simplest and widest used mode, cells are violently shaken with 500-μm diameter beads in cold buffers using a basic vortex apparatus in cycles alternating shaking and ice-cooling phases ([38, 138] among others). In order to achieve more reliable and reproducible results, programmable equipment is recommended, such as the Tissue Lyser from Qiagen [132] or the FastPrep 24 from MP Biomedicals [102] that accommodate various sample volumes and formats (up to 50ml), or the more sophisticated and expensive grinder series from Dyno Mills [85] that can operate using large volumes both in batch or continuous modes. In addition, pressure-based instruments have also proven efficient for the lysis of *P. pastoris* cells, and besides the well-known French pressure cell press stands a panel of cell disruptors from Constant Systems that can handle from 1 to 20ml of batch samples and more than 500ml/min with the continuous flow models. Alternatively, methods involving glucanase enzymes (e.g., helicase from snail digestive juice; zymolyase or lyticase from microbial sources) can be used to remove the cell wall and give rise to spheroplast preparations that can be easily burst. For cost and practical reasons, however, these methods are obviously not recommended for the lysis of large cell volumes.

Noteworthy, we and others commonly observed that the longer the induction phase, the less efficient the lysis step. This is likely to be related to the very dynamic nature of the cell wall that can adapt to various physiological changes in order to maintain the integrity of yeast cells and prevent them from lysis [139]. This issue is notably to be considered when optimizing the induction time, which should ideally strike a balance between expression level and cell lysis efficiency.
3.7 Conclusions and Future Directions

Overall, P. pastoris appears a very efficient heterologous system for the production of a large panel of membrane proteins. Importantly, a series of various optimizations including gene and vector sequence engineering, host strains, clone selection, culture format and procedures, temperature and time of induction, cell densities, formulation of growth media, and supplementation with stabilizing compounds or chemical chaperones, always prove very helpful for improving expression levels and protein functionality. As an exemplar illustration of such improvements, the functional expression level of a dopaminergic D₂S GPCR was increased from about 1000 receptors per cell for an unmodified receptor up to more than 50000 receptors per cell for an ultimate engineered αFD2SBio receptor [90].

Even if some general tips emerge, the outcome is, however, often a matter of membrane protein-dependent adjustments and successes still often rely on trial-and-error strategies. An improved success rate will certainly be obtained in the coming years with a more rationalized use of P. pastoris, taking advantage of recent breakthroughs. Very recently, the combination of proteomics and genetics brought some mechanistic insights into the biology of recombinant production of membrane proteins both in E. coli [140] and S. cerevisiae [141], and subsequently allowed the engineering of strains presenting a specific task-adapted physiology and enhanced production properties. Such strain evolution strategies have been already applied to P. pastoris, notably for the production of human glycoproteins in glyco-engineered strains [23]. They will be probably further exploited with the recent release of the whole-genome sequence of P. pastoris [9] that is now fully accessible to proteomic analyses and genetic manipulations.

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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| β AR         | β₂-adrenergic receptor |
| ABC          | ATP-binding cassette |
| CBD          | calmodulin binding domain |
| DMSO         | dimethyl sulfoxide |
| GFP          | Green Fluorescent Protein |
| GPCR         | G-protein-coupled receptor |
| ORF          | open reading frame |
| TEV          | tobacco etch virus |
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Abstract:
Among the most widespread, popular, effective, and inexpensive microorganisms developed for heterologous expression, *Pichia pastoris* has become a system of choice not only for the production of cytosoluble and industrially relevant proteins, but also for a growing panel of eukaryotic membrane proteins expressed at levels compatible with structural studies. Up to now indeed, more than 150 different representative membrane proteins have been expressed in *P. pastoris* and led to acquiring of high-resolution structures for a dozen of them, making *P. pastoris* one of the most performant heterologous expression system for the structural studies of eukaryotic membrane proteins. This chapter gives a global overview on (i) how the *P. pastoris* system basically operates, (ii) how it performs for the recombinant expression of membrane proteins, and (iii) the different strategies and tips that can be applied to improve the system.