

**Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris***  
**(review)**

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## **Abstract**

Having decided on yeast as a production host, the choice of species is often the first question any researcher new to the field will ask. With over 500 known species of yeast to date, this could pose a significant challenge. However, in reality, only very few species of yeast have been employed as host organisms for the production of recombinant proteins. The two most widely used, *Saccharomyces cerevisiae* and *Pichia pastoris*, are compared and contrasted here.

**Keywords:** Yeast, host cell, recombinant protein production

## **1. Introduction**

Yeast is a single-celled, eukaryotic microbe that can grow quickly in complex or defined media (doubling times are typically 2.5 h in glucose-containing media) and is easier and less expensive to use for recombinant protein production than insect or mammalian cells (1). These positive attributes make yeast suitable for use in formats ranging from multi-well plates, shake flasks and continuously-stirred tank bioreactors to pilot plant and industrial scale reactors.

The most commonly-employed species in the laboratory are *Saccharomyces cerevisiae* (also known as Baker's or Brewer's yeast) and some methylotrophic yeasts of the *Pichia* genus. In particular, *S. cerevisiae* and *Pichia pastoris*, which have both been genetically characterised (2-4), have been shown to perform the posttranslational disulphide bond formation and glycosylation (5-7) that is crucial for the proper functioning of some recombinant proteins. However, it is important to note that yeast glycosylation does differ from that in mammalian cells: in *S. cerevisiae*, O-linked oligosaccharides contain only mannose moieties, whereas

higher eukaryotic proteins have sialylated O-linked chains. Furthermore *S. cerevisiae* is known to hyperglycosylate N-linked sites, which can result in altered protein binding, activity, and potentially yield an altered immunogenic response in therapeutic applications (8). In *P. pastoris*, oligosaccharides are of much shorter chain length (9) and a strain has been reported that can produce complex, terminally-sialylated or “humanised” glycoproteins (10).

Despite these potential limitations, recombinant pharmaceuticals including insulin (11), interferon-alpha-2a (Reiferon Retard<sup>®</sup>) and vaccines against hepatitis B virus (Hepavax-Gene and Engerix-B<sup>®</sup>) (12) and Human papilloma virus (Gardasil<sup>®</sup>) (13, 14) have all been produced in yeasts for commercial use, demonstrating the importance of yeast as a host organism to the pharmaceutical industry. The benefits and limitations of using *S. cerevisiae* and *P. pastoris* on a laboratory scale are addressed here and specific examples of their uses for the production of both soluble and membrane proteins are discussed.

## **2. *Saccharomyces cerevisiae***

*S. cerevisiae* is a single-celled, budding yeast, approximately 5–10 µm in size. Whilst it is commonly associated with the brewing and baking industries on account of its ability to produce ethanol and carbon dioxide, it is also the most widely-studied eukaryotic organism. The USA’s Food and Drug Administration (FDA) award of “generally recognized as safe” (GRAS) status to *S. cerevisiae* means that it is the most frequently-used species of yeast for the production of many functional proteins. These include several soluble antibody fragments and fusions (15-19) as well as membrane protein drug targets such as G protein-coupled receptors (20-25), ABC transporters (9, 26) and drug resistance proteins (27).

### **2.1 Microbiology**

(Insert Tables 1 and 2 here)

The microbiology of *S. cerevisiae* is well understood and has been extensively reviewed elsewhere (28). In essence, it can grow both aerobically and anaerobically on a variety of carbon sources, is able to use ammonia or urea as a nitrogen source and also requires phosphorus and sulphur in its growth media. Certain metals such as calcium, iron, magnesium and zinc enhance its growth (29, 30): **Tables 1 and 2** summarises typical growth media for *S. cerevisiae*. In culture it has a relatively short generation time, doubling its cell density approximately every 1.5–2.5 h at its preferred growth temperature of 30 °C.

The ability of *S. cerevisiae* to produce ethanol hints at its unusual metabolism: in most eukaryotes, oxygen depletion controls the switch from a respiratory to a fermentative metabolism, but in *S. cerevisiae* this switch also occurs in response to a change in the external concentration of a fermentable carbon source such as glucose (31). During the type of aerobic batch cultivation on glucose often performed on a laboratory scale, *S. cerevisiae* displays a biphasic growth pattern (**Figure 1**). In the first respiro-fermentative phase, most of the glucose is converted to ethanol (32), which is subsequently consumed to produce carbon dioxide and water in the second phase. This has evolutionary advantages for *S. cerevisiae*, as the ethanol production phase is associated with a higher specific growth rate than the respiratory phase, providing a competitive advantage over other non-ethanol-producing organisms. Maximum recombinant protein yields are usually highest before yeast cells reach the end of this respiro-fermentative phase, before the so-called “diauxic shift” (**Figure 1**) into the respiratory phase (33). Consequently *S. cerevisiae* cells are typically harvested just before this diauxic shift in a protein production experiment, which can be readily assessed by

monitoring the off-gas profile or the glucose concentration in the culture (24, 33). A respiratory strain of *S. cerevisiae*, TM6\*, has been reported to have improved yield properties for both recombinant soluble and membrane proteins on account of its altered metabolism (24, 33). Its improved biomass yields, which are achieved at the expense of ethanol production, result in an increased volumetric yield of recombinant protein (34).

(Insert Figure 1 here)

## 2.2 Genetics

*S. cerevisiae* was the first eukaryote to have its complete genome sequenced (2). The data are publicly available from the *Saccharomyces* Genome Database (SGD; [www.yeastgenome.org](http://www.yeastgenome.org)), which is a scientific database of yeast molecular biology and genetics. The SGD provides detailed descriptions of the phenotypes of many mutant *S. cerevisiae* strains, many of which may have potential as protein production hosts, with links to supporting literature. Importantly, the SGD is fully annotated and is continually updated.

Resources such as the SGD have facilitated an increased understanding of *S. cerevisiae* in general and, more specifically in the context of this discussion, of recombinant protein production in yeast cells. For example, since the production of high yields of functional recombinant proteins, particularly membrane proteins, remains a significant challenge we and others (35, 36) have focussed on characterising the cellular response to recombinant protein production. Identifying specific genes that exhibit an altered transcriptional profile, when the cell produces high yields of functional recombinant proteins, has been used to guide subsequent engineering of high yielding strains (24, 33, 37). This approach is strengthened by the availability of a complete set of single, non-essential gene deletion strains

(EUROSCARF) as well as a strain collection of tetracycline-regulated essential genes (Open Biosystems). We demonstrated that increased yields of recombinant proteins can be achieved when specific members of these collections are used as host organisms (37). This permits the production of a recombinant protein to be compared in multiple strains simultaneously whilst gaining an improved knowledge of the molecular pathways involved in producing the protein.

### **2.3 Molecular biology**

The DNA sequence encoding the target protein of interest is typically amplified *via* PCR either from genomic DNA or cDNA and then cloned into a suitable expression plasmid with or without signal sequences and fusion partners (see **Chapters 3 and 4**). Plasmids for *S. cerevisiae* can be sub-divided into three categories: low-copy number replicating plasmids; multi-copy number replicating plasmids and integrative plasmids (38, 39). These can, in turn, contain a range of different promoters of varying strengths that are either inducible or constitutive (**Table 3**). This means that a variety of different options can be tested in order to optimise the most suitable regime for recombinant protein production. However, it is important to ensure the stability of any transformants generated. For example, autonomous plasmids can be relatively unstable, yielding a heterogenous population of transformants that routinely require screening for the desired expression level, as well as being prone to genetic loss upon cell division. Furthermore high copy number plasmids may also result in expression levels that can overwhelm the host's post-translational and secretory pathways, yielding misfolded and degraded protein. It may be possible to overcome some of these problems by integrating the expression cassette into the genome, thereby increasing its genetic stability (38, 39).

(Insert Table 3 here)

### **3. *Pichia pastoris***

*P. pastoris* is used increasingly as the host cell of choice because of its ability to produce high yields of properly-folded proteins in exceptionally high density cultures. To achieve the highest possible biomass yields, it must be cultured in fully-controlled, continuously-stirred tank bioreactors (40). Its emergence as an alternative to *S. cerevisiae* is exemplified by the variety of heterologous proteins it has been used to produce in high yields, ranging from tetanus toxin and mouse epidermal growth factor (41-43) to membrane proteins including human ABC transporters, aquaporins and tetraspanins (44-46). A number of different protease deficient strains are also available (SMD1163, 1165 and 1168) that have been shown to exhibit reduced proteolysis of some recombinant proteins (47-50).

#### **3.1 Microbiology**

*P. pastoris* has a respiratory metabolism (Figure 1) and can be cultured to exceptionally high cell densities (hundreds of grams per litre) on glycerol-containing media (Tables 1 and 2), often yielding a culture resembling a paste at the end of an experiment (43, 51). The development of a respiratory strain of *S. cerevisiae* (TM6\*) (24, 52) has gone some way to permitting similarly high cell density cultures of *S. cerevisiae*. However, while high cell density cultures are very attractive for increasing volumetric yields, productivity does not necessarily increase linearly with increased biomass yields, and in some situations may actually decrease (40, 53). For this reason, the ability to increase “per cell” yields is an area of active research, not only in *P. pastoris* but also in other host cells (34).

*P. pastoris* is a methylotroph, which has two endogenous copies of the *AOX* gene; *AOX1* expression accounts for more than 90 % of the enzyme in the cell whilst *AOX2* expression constitutes less than 10 % (see **Chapter 15**). It is these genes that permit the utilisation of methanol as a carbon source: using the *Pichia* Expression Kit marketed by Invitrogen Corporation, it is possible to use the *AOX* promoter to control heterologous gene expression, which is induced in the presence of methanol and repressed by glucose or glycerol (**51**). The careful control of the methanol induction regime is central to increasing yields per cell (**40**). Methanol metabolism is highly dependent on oxygen availability within the culture and it is widely accepted that the dissolved oxygen concentration (DO) should be maintained above 20 % (**54**). This regime has been successfully employed to produce many different soluble and membrane proteins (**40, 44, 46, 53, 55-57**).

Despite methanol induction being robust and tightly regulated, the potential risks associated with methanol use, such as its toxicity to cells at concentrations above 5 g L<sup>-1</sup> and its volatility, have led researchers to investigate alternative promoters that do not require the use of methanol (**58**). Constitutive expression of heterologous genes can be achieved when cloned downstream of the glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter (**59, 60**), whilst strong induction via the formaldehyde dehydrogenase (*FLDI*) promoter has also been reported in the presence of methylamine as well as methanol (**61**).

Like *S. cerevisiae*, *P. pastoris* is capable of producing disulfide bonded and glycosylated proteins (**62**). However, the glycosylation pattern is different in *P. pastoris* compared with *S. cerevisiae* (**63**): in *P. pastoris* N-linked oligosaccharides are usually no more than 20 residues in length compared with 50–150 residues in *S. cerevisiae*. In addition, *P. pastoris* lacks the



mannosyl transferase which yields immunogenic  $\alpha$ -1, 3-linked mannosyl terminal linkages in *S. cerevisiae* (64).

### 3.2 Genetics

*P. pastoris* is not considered to be as genetically amenable as *S. cerevisiae*, despite the fact that a genomic sequence of the GS115 strain has been commercially available since the mid 2000's. This is partly on account of a restrictive user contract that required all sequence information to be confidentially maintained. Despite this, genetic advances have been made as highlighted by the development of a "humanised" *P. pastoris* strain (10) capable of replicating the most essential glycosylation pathways found in mammalian cells and permitting the production of active recombinant erythropoietin. The open-access publication of the GS115 (3) and DSMZ 70382 (4) genomes in 2009 and their respective annotation at <http://bioinformatics.psb.ugent.be/webtools/bogas/> and <http://www.pichiagenome.org> should make a significant impact in this area, as both sites permit free access to view the genomic sequences and use sequence resource software (65).

### 3.3 Molecular biology

As for *S. cerevisiae*, the target protein's DNA sequence is often PCR amplified from genomic DNA or cDNA and cloned into an expression plasmid with or without signal sequences and fusion partners. The most widely-used *P. pastoris* expression vectors are designed to be maintained as stable, integrative elements in its genome (see **Chapter 3**). Examples include Invitrogen's pPIC and pGAPZ series of vectors (**Table 3**). However, transformants often exhibit heterogeneous expression levels and this necessitates the screening of many colonies to isolate high-yielding clones (see **Chapter 7**). The limited number of episomal plasmids for *P. pastoris* to date has been predominantly due to plasmid instability during replication

(66). Those that are available (**Table 3**) often utilise the constitutive *GAP* promoter (67, 68) and require the addition of selective antibiotic to maintain the vector. With the advent of open access genomic data it is hoped that there will be an increase in the number of episomal vectors that contain different auxotrophic markers for selection.

#### **4. Which species should I choose?**

There are benefits and drawbacks to using both *S. cerevisiae* and *P. pastoris* as hosts for recombinant protein production. For the production of secreted proteins, *P. pastoris* may be the best choice on account of its limited endogenous protein secretion and the number of different protease deficient strains available. However, the full benefit of *P. pastoris* will only be achieved if it is cultured under strictly-defined conditions, usually only achievable in continuously-stirred tank bioreactors. Therefore the optimal use of *P. pastoris* may require a more long term investment of time and equipment resources than for *S. cerevisiae*. In contrast *S. cerevisiae* provides a much wider range of resources (both strains and expression vectors) and is supported by a much more extensive literature than *P. pastoris*. Consequently, projects requiring a range of strains may benefit from using *S. cerevisiae* as the host. In our laboratory, we often start with *P. pastoris* and if the production is not straightforward, turn to *S. cerevisiae* to troubleshoot, thereby benefitting from the best attributes of the two hosts.

#### **5. References**

1. Bill, R. M. (2001) Yeast - a panacea for the structure-function analysis of membrane proteins?, *Current Genetics* **40**, 157-171.
2. Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W.,

- Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S. G. (1996) Life with 6000 genes, *Science* **274**, 546-567.
3. De Schutter, K., Lin, Y. C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouze, P., de Peer, Y. V. and Callewaert, N. (2009) Genome sequence of the recombinant protein production host *Pichia pastoris*, *Nat Biotechnol* **27**, 561-566.
  4. Mattanovich, D., Graf, A., Stadlmann, J., Dragosits, M., Redl, A., Maurer, M., Kleinheinz, M., Sauer, M., Altmann, F. and Gasser, B. (2009) Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*, *Microb Cell Fact* **8**, 29
  5. Demain, A. L. and Vaishnav, P. (2009) Production of recombinant proteins by microbes and higher organisms, *Biotechnol Adv* **27**, 297-306.
  6. Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J. L., Vazquez, E. and Villaverde, A. (2009) Microbial factories for recombinant pharmaceuticals, *Microb Cell Fact* **8**, 17.
  7. Jigami, Y. (2008) Yeast glycobiology and its application, *Bioscience Biotechnol Biochem* **72**, 637-648.
  8. Gerngross, T. U. (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi, *Nat Biotechnol* **22**, 1409-1414.
  9. Bretthauer, R. K. and Castellino, F. J. (1999) Glycosylation of *Pichia pastoris*-derived proteins, *Biotechnol Appl Biochem* **30**, 193-200.
  10. Hamilton, S. R., Davidson, R. C., Sethuraman, N., Nett, J. H., Jiang, Y. W., Rios, S., Bobrowicz, P., Stadheim, T. A., Li, H. J., Choi, B. K., Hopkins, D., Wischnewski, H., Roser, J., Mitchell, T., Strawbridge, R. R., Hoopes, J., Wildt, S. and Gerngross, T. U. (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins, *Science* **313**, 1441-1443.

11. Kjeldsen, T., Ludvigsen, S., Diers, I., Balschmidt, P., Sørensen, A. R. and Kaarsholm, N. C. (2002) Engineering-enhanced protein secretory expression in yeast with application to insulin, *J Biol Chem* **277**, 18245-18248.
12. Andre, F. E. and Safary, A. (1987) Summary of clinical findings on engerix-b, a genetically engineered yeast-derived Hepatitis-B vaccine, *Postgrad Med J* **63**, 169-178.
13. Siddiqui, M. A. A. and Perry, C. M. (2006) Human papillomavirus quadrivalent (types 6, 11, 16, 18) recombinant vaccine (Gardasil (R)): Profile report, *Biodrugs* **20**, 313-316.
14. Siddiqui, M. A. A. and Perry, C. M. (2006) Human papillomavirus quadrivalent (types 6, 11, 16, 18) recombinant vaccine (Gardasil (R)), *Drugs* **66**, 1263-1271.
15. Gasser, B. and Mattanovich, D. (2007) Antibody production with yeasts and filamentous fungi: on the road to large scale?, *Biotechnol Lett* **29**, 201-212.
16. Hackel, B. J., Huang, D. G., Bubozy, J. C., Wang, X. X. and Shusta, E. V. (2006) Production of soluble and active transferrin receptor-targeting single-chain antibody using *Saccharomyces cerevisiae*, *Pharmaceut Res* **23**, 790-797.
17. Evans, L., Hughes, M., Waters, J., Cameron, J., Dodsworth, N., Tooth, D., Greenfield, A. and Sleep, D. (2010) The production, characterisation and enhanced pharmacokinetics of scFv-albumin fusions expressed in *Saccharomyces cerevisiae*, *Protein Expr Purif* **73**, 113-124.
18. Frenken, L. G. J., van der Linden, R. H. J., Hermans, P. W. J. J., Bos, J. W., Ruuls, R. C., de Geus, B. and Verrips, C. T. (2000) Isolation of antigen specific Llama V-HH antibody fragments and their high level secretion by *Saccharomyces cerevisiae*, *J Biotechnol* **78**, 11-21.

19. Edqvist, J., Keranen, S., Penttila, M., Straby, K. B. and Knowles, J. K. C. (1991) Production of Functional Igm Fab Fragments by *Saccharomyces cerevisiae*, *J Biotechnol* **20**, 291-300.
20. Liitti, S., Matikainen, M. T., Scheinin, M., Glumoff, T. and Goldman, A. (2001) Immunoaffinity purification and reconstitution of human alpha(2)-adrenergic receptor subtype C2 into phospholipid vesicles, *Protein Expr Purif* **22**, 1-10.
21. Huang, H. J., Liao, C. F., Yang, B. C. and Kuo, T. T. (1992) Functional Expression of Rat M5 Muscarinic Acetylcholine-Receptor in Yeast, *Biochem Biophys Res Commun* **182**, 1180-1186.
22. Price, L. A., Strnad, J., Pausch, M. H. and Hadcock, J. R. (1996) Pharmacological characterization of the rat A(2a) adenosine receptor functionally coupled to the yeast pheromone response pathway, *Mol Pharmacol* **50**, 829-837.
23. Joubert, O., Nehme, R., Bidet, M. and Mus-Veteau, I. (2010) Heterologous expression of human membrane receptors in the yeast *Saccharomyces cerevisiae*, *Heterologous Expression of Membrane Proteins: Methods and Protocols*, 87-103.
24. Ferndahl, C., Bonander, N., Logez, C., Wagner, R., Gustafsson, L., Larsson, C., Hedfalk, K., Darby, R. A. J. and Bill, R. M. (2010) Increasing cell biomass in *Saccharomyces cerevisiae* increases recombinant protein yield: the use of a respiratory strain as a microbial cell factory, *Microb Cell Fact* **9**, 47.
25. Kapat, A., Jaakola, V. P., Heimo, H., Liitti, S., Heikinheimo, P., Glumoff, T. and Goldman, A. (2000) Production and purification of recombinant human alpha 2C2 adrenergic receptor using *Saccharomyces cerevisiae*, *Bioseparation* **9**, 167-172.
26. Duman, J. G., Miele, R. G., Liang, H., Grella, D. K., Sim, K. L., Castellino, F. J. and Bretthauer, R. K. (1998) O-Mannosylation of *Pichia pastoris* cellular and recombinant proteins, *Biotechnol Appl Biochem* **28**, 39-45.

27. Miele, R. G., Castellino, F. J. and Bretthauer, R. K. (1997) Characterization of the acidic oligosaccharides assembled on the *Pichia pastoris*-expressed recombinant kringle 2 domain of human tissue-type plasminogen activator, *Biotechnol Appl Biochem* **26**, 79-83.
28. Barnett, J. A. and Barnett, L. (2011) *Yeast Research: A Historical Overview*, ASM Press, Herndon, VA.
29. Treco, D. A. and Lundblad, V. (2001) Preparation of yeast media, *Current Protocols in Molecular Biology Chapter 13*, Unit13.1
30. Curran, B. P., Bugeja, V.. (2006) Basic investigations in *Saccharomyces cerevisiae*, *Methods in Molecular Biology* **313**, 1-13.
31. Bonander, N., Ferndahl, C., Mostad, P., Wilks, M. D., Chang, C., Showe, L., Gustafsson, L., Larsson, C. and Bill, R. M. (2008) Transcriptome analysis of a respiratory *Saccharomyces cerevisiae* strain suggests the expression of its phenotype is glucose insensitive and predominantly controlled by *Hap4*, *Cat8* and *Mig1*, *BMC Genomics* **9**, 365.
32. Verduyn, C., Zomerdijk, T. P. L., Dijken, J. P. and Scheffers, W. A. (1984) Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode, *Appl Microbiol Biotechnol* **19**, 181-185.
33. Bonander, N., Hedfalk, K., Larsson, C., Mostad, P., Chang, C., Gustafsson, L. and Bill, R. M. (2005) Design of improved membrane protein production experiments: Quantitation of the host response, *Prot Sci* **14**, 1729-1740.
34. Bawa, Z., Bland, C. E., Bonander, N., Bora, N., Cartwright, S. P., Clare, M., Conner, M. T., Darby, R. A., Dilworth, M. V., Holmes, W. J., Jamshad, M., Routledge, S. J., Gross, S. R. and Bill, R. M. (2011) Understanding the yeast host cell response to recombinant membrane protein production, *Biochem Soc Trans* **39**, 719-723.

35. Wang, H., Prorok, M., Bretthauer, R. K. and Castellino, F. J. (1997) Serine-578 is a major phosphorylation locus in human plasma plasminogen, *Biochemistry* **36**, 8100-8106.
36. Ren, J., Castellino, F. J. and Bretthauer, R. K. (1997) Purification and properties of alpha-mannosidase II from Golgi-like membranes of baculovirus-infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells, *Biochem J* **324**, 951-956.
37. Bonander, N., Darby, R. A. J., Grgic, L., Bora, N., Wen, J., Brogna, S., Poyner, D. R., O'Neill, M. A. A. and Bill, R. M. (2009) Altering the ribosomal subunit ratio in yeast maximizes recombinant protein yield, *Microb Cell Fact* **8**, 10.
38. Schneider, J. C. and Guarente, L. (1991) Vectors for expression of cloned genes in yeast: Regulation, overproduction and underproduction, *Methods Enzymol* **194**, 373-388.
39. Zhang, Z., Moo-Young, M. and Chisti, Y. (1996) Plasmid stability in recombinant *Saccharomyces cerevisiae*, *Biotechnol Adv* **14**, 401-435.
40. Holmes, W. J., Darby, R. A. J., Wilks, M. D. B., Smith, R. and Bill, R. M. (2009) Developing a scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime, *Microb Cell Fact* **8**, 35.
41. Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K. and Romanos, M. A. (1991) High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene, *Bio-Technology* **9**, 455-460.
42. Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K. and Henwood, C. A. (1991) Production of mouse epidermal growth-factor in yeast- high-level secretion using *Pichia pastoris* strains containing multiple gene copies, *Gene* **105**, 205-212.

43. Macauley-Patrick, S., Fazenda, M. L., McNeil, B. and Harvey, L. M. (2005) Heterologous protein production using the *Pichia pastoris* expression system, *Yeast* **22**, 249-270.
44. Rosenberg, M. F., Bikadi, Z., Chan, J., Liu, X. P., Ni, Z. L., Cai, X. K., Ford, R. C. and Mao, Q. C. (2010) The human breast cancer resistance protein (BCRP/ABCG2) shows conformational changes with mitoxantrone, *Structure* **18**, 482-493.
45. Urbatsch, I. L., Wilke-Mounts, S., Gimi, K. and Senior, A. E. (2001) Purification and characterization of n-glycosylation mutant mouse and human p-glycoproteins expressed in *Pichia pastoris* cells, *Arch Biochem Biophys* **388**, 171-177.
46. Jamshad, M., Rajesh, S., Stamataki, Z., McKeating, J. A., Dafforn, T., Overduin, M. and Bill, R. M. (2008) Structural characterization of recombinant human CD81 produced in *Pichia pastoris*, *Prot Expr Purif* **57**, 206-216.
47. Grunewald, S., Haase, W., Molsberger, E., Michel, H. and Reilander, H. (2004) Production of the human D-2S receptor in the methylotrophic yeast *P. pastoris*, *Receptors Channels* **10**, 37-50.
48. Shi, X. L., Feng, M. Q., Shi, J., Shi, Z. H. A., Zhong, J. A. and Zhou, P. (2007) High-level expression and purification of recombinant human catalase in *Pichia pastoris*, *Prot Expr Purif* **54**, 24-29.
49. Ogunjimi, A. A., Chandler, J. M., Gooding, C. M., Recinos, A. and Choudary, P. V. (1999) High-level secretory expression of immunologically active intact antibody from the yeast *Pichia pastoris*, *Biotechnol Lett* **21**, 561-567.
50. Andre, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R. and Reinhart, C. (2006) Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen, *Prot Sci* **15**, 1115-1126.



51. Cregg, J. M., Cereghino, J. L., Shi, J. Y. and Higgins, D. R. (2000) Recombinant protein expression in *Pichia pastoris*, *Mol Biotechnol* **16**, 23-52.
52. Otterstedt, K., Larsson, C., Bill, R. M., Stahlberg, A., Boles, E., Hohmann, S. and Gustafsson, L. (2004) Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*, *EMBO Rep* **5**, 532-537.
53. Singh, S., Hedley, D., Kara, E., Gras, A., Iwata, S., Ruprecht, J., Strange, P. G. and Byrne, B. (2010) A purified C-terminally truncated human adenosine A2A receptor construct is functionally stable and degradation resistant, *Prot Expr Purif* **74**, 80-87.
54. Li, P. Z., Anumanthan, A., Gao, X. G., Ilangoan, K., Suzara, V. V., Duzgunes, N. and Renugopalakrishnan, V. (2007) Expression of recombinant proteins in *Pichia pastoris*, *Appl Biochem Biotechnol*. **142**, 105-124.
55. Yinliang, C., Cino, J., Hart, G., Freedman, D., White, C. and Komives, E. A. (1997) High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process, *Process Biochem* **32**, 107-111.
56. Jin, H., Liu, G., Ye, X., Duan, Z., Li, Z. and Shi, Z. (2010) Enhanced porcine interferon- $\alpha$  production by recombinant *Pichia pastoris* with a combinational control strategy of low induction temperature and high dissolved oxygen concentration, *Biochem Eng J* **52**, 91-98.
57. Fraser, N. J. (2006) Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies, *Prot Expr Purif* **49**, 129-137.
58. Cos, O., Ramon, R., Montesinos, J. L. and Valero, F. (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review, *Microb Cell Fact* **5**, 17.

59. Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V. and Cregg, J. M. (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter, *Gene* **186**, 37-44.
60. Kim, S. J., Lee, J. A., Kim, Y. H. and Song, B. K. (2009) optimization of the functional expression of coprinus cinereus peroxidase in *Pichia pastoris* by varying the host and promoter, *J Microbiol Biotechnol* **19**, 966-971.
61. Resina, D., Cos, O., Ferrer, P. and Valero, F. (2005) Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter, *Biotechnol Bioeng* **91**, 760-767.
62. Daly, R. and Hearn, M. T. W. (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production, *J Mol Recog* **18**, 119-138.
63. Dale, C., Allen, A. and Fogerty, S. (1999) *Pichia pastoris*: A eukaryotic system for the large-scale production of biopharmaceuticals, *Biopharm* **12**, 36-40.
64. Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*, *Nat Biotechnol* **11**, 905-910.
65. Mattanovich, D., Callewaert, N., Rouze, P., Lin, Y. C., Graf, A., Redl, A., Tiels, P., Gasser, B. and De Schutter, K. (2009) Open access to sequence: Browsing the *Pichia pastoris* genome, *Microb Cell Fact* **8**, 53.
66. Cregg, J. M., Barringer, K. J., Hessler, A. Y. and Madden, K. R. (1985) *Pichia-pastoris* as a host system for transformations, *Mol Cell Biol* **5**, 3376-3385.
67. Lee, C. C., Williams, T. G., Wong, D. W. S. and Robertson, G. H. (2005) An episomal expression vector for screening mutant gene libraries in *Pichia pastoris*, *Plasmid* **54**, 80-85.

68. Choi, S. G., Hong, I. P. and Anderson, S. (2006) Evaluation of a new episomal vector based on the GAP promoter for structural genomics in *Pichia pastoris*, *J Microbiol Biotechnol* **16**, 1362-1368.

## Figure legend

**Figure 1:** (a) A representative CO<sub>2</sub> gas profile recorded in our laboratory, using a Tandem gas analyser, for a glucose-limited *S. cerevisiae* culture grown in a 2L Applikon bioreactor. The solid line shows the production of CO<sub>2</sub> (%) and the dashed line shows the depletion of glucose (mmol). The respiro-fermentative and respiration phases are indicated, as is the diauxic shift between the 2 phases. (b) A CO<sub>2</sub> gas profile recording for a *P. pastoris* glycerol fed-batch bioreactor culture induced at 28 h with a limiting amount of methanol. The solid line shows the production of CO<sub>2</sub> (%) and the dashed line shows the optical density of the culture.

**Table 1:** Composition of typical media for culturing *S. cerevisiae* and *P. pastoris*

<i>S. cerevisiae</i> medium	Components (L <sup>-1</sup> )
YPD (rich medium)	10 g Bacto yeast extract 20 g Bacto peptone 20 g glucose
YPG (rich medium with non-fermentable carbon source)	10 g Bacto yeast extract 20 g Bacto peptone 30 mL glycerol
CSM (complete synthetic medium)	1.7 g Bacto yeast nitrogen base (without amino acids) 5 g ammonium sulphate 20 g glucose 100 mL 10× amino acid solution (see Table 2)
2× CBS (Centralbureau voor Schimmelcultures medium)	10 g ammonium sulphate 6 g potassium dihydrogen phosphate 1 g magnesium sulphate heptahydrate 20 g glucose 100 mL 1 M MES, pH 6 200 mL 10× amino acid solution (see Table 2) 2 mL vitamin solution (see Table 2) 2 mL trace element solution (see Table 2)
<i>P. pastoris</i> medium	Components (L <sup>-1</sup> )
BMGY (buffered glycerol-complex medium)	10 g Bacto yeast extract 20 g Bacto peptone 100 mL 10× YNB (see Table 2) 100 mL 1 M potassium phosphate (pH 6) 2 mL 500× biotin (see Table 2) 100 mL 10× glycerol (see Table 2)
BMMY (buffered methanol-complex medium)	10 g Bacto yeast extract 20 g Bacto peptone 100 mL 10× YNB (see Table 2) 100 mL 1 M potassium phosphate (see Table 2) 2 mL 500× biotin (see Table 2) 100 mL 10× methanol (see Table 2)

BSM (basal salts medium)

26.7 mL phosphoric acid  
0.93 g calcium sulphate  
18.2 g potassium sulphate  
14.9 g magnesium sulphate heptahydrate  
4.13 g potassium hydroxide  
40 g glycerol  
4.35 mL PTM<sub>1</sub> Salts (see Table 2)

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**Table 2:** Composition of stock solutions required to prepare media in Table 1

<i>S. cerevisiae</i> medium stocks	Components (L <sup>-1</sup> )
10× amino acid solution	200 mg L-adenine hemisulphate 200 mg L-arginine hydrochloride 200 mg L-histidine hydrochloride monohydrate 300 mg L-isoleucine 1000 mg L-leucine 300 mg L-lysine hydrochloride 200 mg L-methionine 500 mg L-phenylalanine 2000 mg L-threonine 200 mg L-tryptophan 300 mg L-tyrosine 200 mg L-uracil 1500 mg L-valine
Vitamin solution (filter sterilised)	0.05 g biotin 1 g calcium D-pantothenate 1 g nicotinic acid 25 g myo-inositol 1 g thiamine hydrochloride 1 g pyridoxol hydrochloride 0.2 g D-amino benzoic acid
Trace element solution	15 g EDTA 4.5 g zinc sulphate heptahydrate 1 g magnesium chloride tetrahydrate 0.3 g cobalt (II) chloride hexahydrate 0.3 g copper (II) sulphate pentahydrate 0.4 g sodium molybdate dihydrate 4.5 g calcium chloride dihydrate 3 g iron sulphate heptahydrate 1 g boric acid 0.1 g potassium iodide
<i>P. pastoris</i> medium stocks	Components (L <sup>-1</sup> )
10× YNB (filter sterilised)	34 g yeast nitrogen base without ammonium sulphate and amino acids 100 g ammonium sulphate
500× biotin (filter sterilised)	200 mg biotin
1 M potassium phosphate, pH6	868 mL 1 M KH <sub>2</sub> PO <sub>4</sub> ; 132 ml 1 M K <sub>2</sub> HPO <sub>4</sub>

(adjust to pH 6 with KOH and phosphoric acid)

10× glycerol (10 %)

100 mL glycerol

10× methanol (5 %; filter sterilised)

50 mL methanol

PTM<sub>1</sub> salts (filter sterilised)

6 g cupric sulphate pentahydrate

0.08 g sodium iodide

3 g manganese sulphate monohydrate

0.2 g sodium molybdate dihydrate

0.02 g boric acid

0.5 g cobalt chloride

20 g zinc chloride

65 g ferrous sulphate heptahydrate

0.2 g biotin

5 mL sulphuric acid

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**Table 3:** Examples of expression vectors available for *S. cerevisiae* and *P. pastoris*

<b>Vector</b>	<b>Yeast</b>	<b>Selection marker</b>	<b>Promoter</b>	<b>Expression</b>	<b>Episomal or integrative</b>
pYX212	<i>S. cerevisiae</i>	<i>URA3</i>	<i>TPI1</i>	Constitutive	Episomal
pYX222	<i>S. cerevisiae</i>	<i>HIS3</i>	<i>TPI1</i>	Constitutive	Episomal
pYES2	<i>S. cerevisiae</i>	<i>URA3</i>	<i>GAL1</i>	Inducible	Episomal
pVTU260	<i>S. cerevisiae</i>	<i>URA3</i>	<i>ADHI</i>	Constitutive	Episomal
YEpCTHS	<i>S. cerevisiae</i>	<i>Ampicilin</i>	<i>CUP1</i>	Inducible	Episomal
pPICZ	<i>P. pastoris</i>	<i>Zeocin</i>	<i>AOX1</i>	Inducible	Integrative
pGAPZ	<i>P. pastoris</i>	<i>Zeocin</i>	<i>GAP</i>	Constitutive	Integrative
pBGP1	<i>P. pastoris</i>	<i>Zeocin</i>	<i>GAP</i>	Constitutive	Episomal
pGAPZ-E	<i>P. pastoris</i>	<i>Zeocin</i>	<i>GAP</i>	Constitutive	Episomal

**Figure 1**

