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®) and vaccines against hepatitis B virus (Hepavax-Gene and Engerix-B®) (***12***) and Human papilloma virus (Gardasil®) (***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), 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 someway 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^{-1} 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 (*FLD1*) 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 α-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, tranformants 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

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Figure legend

Figure 1: (a) A representative CO_2 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_2 (%) 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_2 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_2 (%) and the dashed line shows the optical density of the culture.

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

***S. cerevisiae* medium**	**Components (L^{-1})**
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)
***P. pastoris* medium**	**Components (L^{-1})**
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_1 Salts (see Table 2)

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

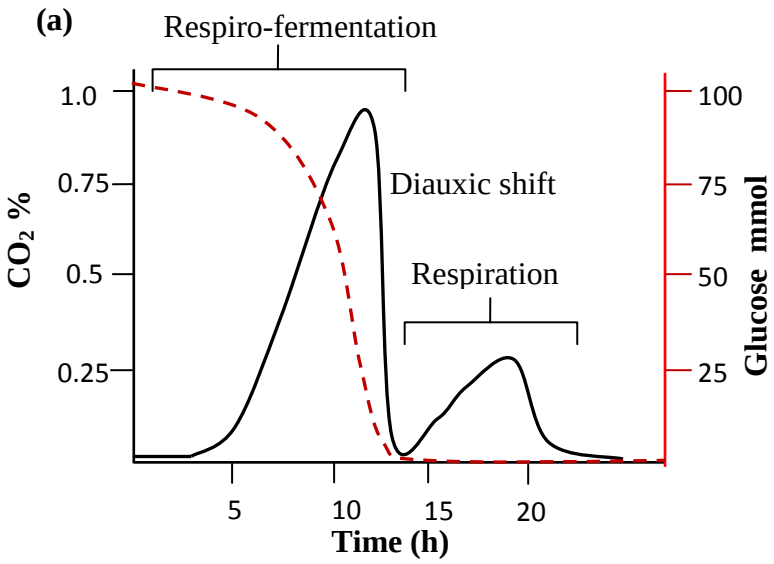
***S. cerevisiae* medium stocks**	**Components (L^{-1})**
10× amino acid solution	200 mg L-adenine hemisulphate 200 mg L-arginine hydrochloride 200 mg L-histidine hydrochloride monohydrate 300 mg L-isoleucine 1000 mg L-leucine 300 mg L-lysine hydrochloride 200 mg L-methionine 500 mg L-phenylalanine 2000 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 colbalt (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
***P. pastoris* medium stocks**	**Components (L^{-1})**
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_2PO_4; 132 ml 1 M K_2HPO_4

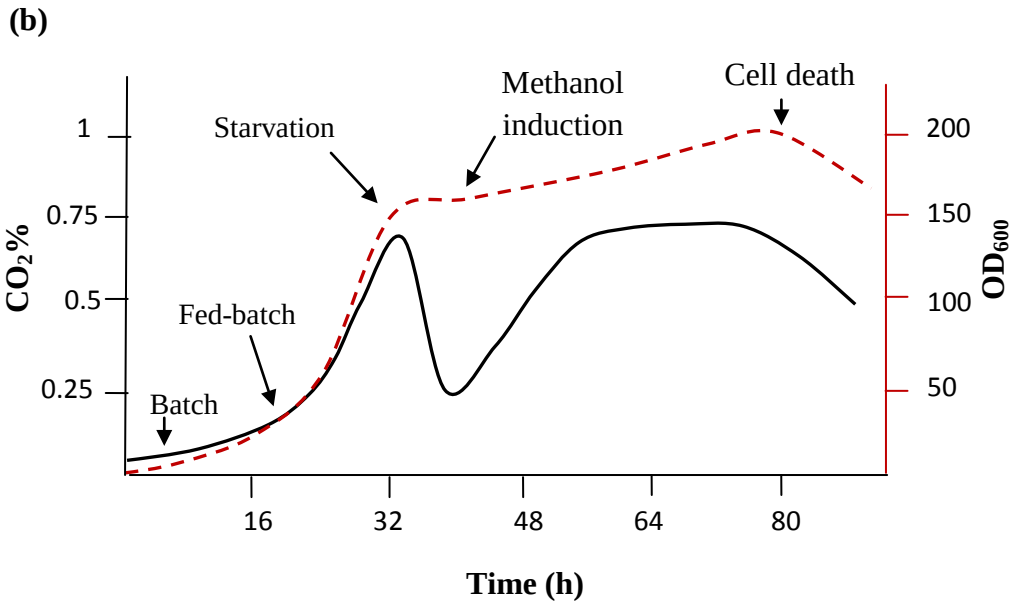
	(adjust to pH 6 with KOH and phosphoric acid)
10× glycerol (10 %)	100 mL glycerol
10× methanol (5 %; filter sterilised)	50 mL methanol
PTM_1 salts (filter sterilised)	6 g cupric sulphate pentahydrate 0.08 g sodium iodide 3 g manganese sulphate monohydrate 0.2 g sodium molybdate dihydrate 0.02 g boric acid 0.5 g cobalt chloride 20 g zinc chloride 65 g ferrous sulphate heptahydrate 0.2 g biotin 5 mL sulphuric acid

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

Vector	Yeast	Selection marker	Promoter	Expression	Episomal or integrative
pYX212	*S. cerevisiae*	*URA3*	*TPI1*	Constitutive	Episomal
pYX222	*S. cerevisiae*	*HIS3*	*TPI1*	Constitutive	Episomal
pYES2	*S. cerevisiae*	*URA3*	*GAL1*	Inducible	Episomal
pVTU260	*S. cerevisiae*	*URA3*	*ADH1*	Constitutive	Episomal
YEpCTHS	*S. cerevisiae*	*Ampicilin*	*CUP1*	Inducible	Episomal
pPICZ	*P. pastoris*	*Zeocin*	*AOX1*	Inducible	Integrative
pGAPZ	*P. pastoris*	*Zeocin*	*GAP*	Constitutive	Integrative
pBGP1	*P. pastoris*	*Zeocin*	*GAP*	Constitutive	Episomal
pGAPZ-E	*P. pastoris*	*Zeocin*	*GAP*	Constitutive	Episomal

Figure 1


(a)
Respiro-fermentation
1.0
0.75
0.5
0.25
CO2 %
Diauxic shift
Respiration
100
75
50
25
Glucose mmol
5
10
15
20
Time (h)


(b)
Methanol
induction
Cell death
1
Starvation
0.75
0.5
0.25
CO2 %
Fed-batch
Batch
200
150
100
50
OD600
16
32
48
64
80
Time (h)