Production of Humanlike Recombinant Proteins in *Pichia pastoris*

From Expression Vector to Fermentation Strategy

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Ever since Invitrogen of Carlsbad, CA (www.invitrogen.com) acquired the distribution rights in 1993 for the *Pichia pastoris* yeast protein expression system, *P. pastoris* has gained widespread popularity, as witnessed by an increasing number of publications (Figure 1). The expression system was originally developed as a single-cell protein production platform by Philips Petroleum of Bartlesville, OK, and later adapted for heterologous protein expression (**1, 2**). It is freely distributed among academic research laboratories, although its use for commercial production requires a license from the current patent holder, Research Corporation Technologies of Tucson, AZ (www.rctech.com).

Renewed interest in yeast and fungal expression systems in general and the *P. pastoris* expression system in particular has been spurred by a growing demand for scalable and cost-effective humanlike therapeutic protein manufacturing systems. The goal is to achieve high fermentation yields in processes that extend for days instead of the weeks required for mammalian expression systems. Today the *P. pastoris* system is licensed to more than 160 companies in the biotechnology, pharmaceutical, vaccine, animal health, and food industries. More than 500 heterologous proteins have been expressed in this host (**3, 4**).

The first of those proteins entered human clinical trials in 1996, followed by an increasing number of candidate therapeutic proteins and antigens (**5**). All yeast-based biopharmaceutical products currently on the market in the United States and Europe are manufactured in *Saccharomyces cerevisiae*; however, a recombinant DNA hepatitis B vaccine and interferon alpha derived from *P. pastoris* have been marketed in India since 1999 and 2002 respectively by Shanta Biotech (www.shantabiotech.com). Similarly, a recombinant human insulin was approved in India in 2003 and marketed by the joint venture of Shanta Biotech and Biocon (www.biocon.com).

Bipha, a subsidiary of Yoshitomi Pharmaceutical (now Mitsubishi Pharma Corporation) has been manufacturing recombinant Human Serum Albumin (rHSA) since 2000 using *P. pastoris* in its Chitose, Japan, factory. The capacity of this plant is an impressive one million vials of rHSA a year, the eq adually increase capacity to over 40 tons a year (**6**; subsequent references **7–17** are called out in Table 2). The dosage/vial of 12.5 g is extremely high compared with other biopharmaceuticals and truly represents a testimony to the high expression levels of *P. pastoris* and establishment of large-scale fermentation technology: up to 80,000-L reactors.

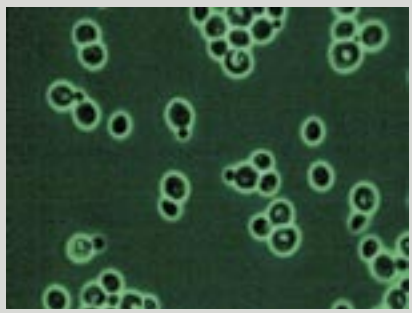


Photo 1 JAMES CREGG, KECK GRADUATE INSTITUTE, CLAREMONT CA (WWW.KGI.EDU)

An Emerging Expression System

P. pastoris is a robust unicellular methylotropic yeast (Photo 1). It combines the unique advantages of prokaryotic growth characteristics and expression levels with the ability to perform posttranslational protein modifications available only in eukaryotic systems (Table 1). Several heterologous proteins expressed in *P. pastoris* have reached expression levels as high as grams per liter. Table 2 lists several therapeutically relevant proteins — including vaccines, antibody fragments, hormones, cytokines, and matrix proteins — along with their achieved expression levels.

In the absence of glycerol, *P. pastoris* uses methanol as its carbon

Product Focus: Therapeutic proteins

Process Focus: Protein expression (production), fermentation, scale-up

Who Should Read: Process development, manufacturing, automation, facility planners

Keywords: *P. pastoris*, methylotropic yeast, methanol utilization phenotypes, Aox1 and Aox2 promoters, glycosylation engineering, batch and fed-batch, DO cascade, methanol sensor

Level: Intermediate

source. A strong alcohol oxidase promoter (alcohol oxidase 1, Aox1) is induced by methanol but repressed in the presence of excess glycerol. The regulation of the Aox1 gene is similar to that of the gal1 gene for *S. cerevisiae* in that the control appears to involve two mechanisms: a catabolyte repression/depression mechanism and an induction mechanism.

Expression Vectors for Humanlike Protein

Several genes in the methanol pathway, including the highly efficient Aox1 promoter, have been cloned (**18–21**) to develop various inducible or constitutive expression vectors that produce recombinant proteins at levels equivalent to those of alcohol oxidase (**22**). In addition, Invitrogen has developed multicopy expression vectors with various selection markers. Both inducible and constitutive expression constructs integrate into the *P. pastoris* genome. As with *S. cerevisiae*, recombination between the expression construct and regions of homology with *P. pastoris* can lead to stably transformed host cells even when harboring multiple gene copies, although the exact mechanism of multiple integration events is not fully understood (**19, 23**).

Expression vectors with signal sequences that allow secretion of heterologous proteins into the culture medium to facilitate downstream processing represent a very interesting prospect for integrated bioprocessing. This is an exciting technological link between cell cultivation and primary downstream unit processing operations up to and including purification in the manufacture of biotechnological products (**24**). The result is a highly productive and cost-effective expression system that has been successfully applied to many recombinant proteins of distinct structure and function (**25–27**).

It should be noted that O- and N-linked glycosylation in native *P. pastoris* differ from glycosylation occurring in mammalian cells. However, recent improvements in glycosylation engineering have produced strains that can express homogenously glycosylated and humanized recombinant proteins (**29–32**). The technological breakthrough in incorporating a limited humanlike glycosylation pathway in *P. pastoris* provides unprecedented opportunities for biopharmaceutical manufacturing. Full humanization of the glycosylation pathway in *P. pastoris* is only one step away from completion (**5**). But the final step encompasses transfer of sialic acid and promises to be challenging because a source of endogenous sialic acid is unknown for yeasts. The importance of terminal sialic acids is highlighted by the often rapid clearance of incompletely sialylated recombinant proteins by the liver.

Figure 1: Publications featuring the *Pichia pastoris* expression system from 1990 to 2005, estimated through linear extrapolation based on actual data for the first nine months (PubMed)

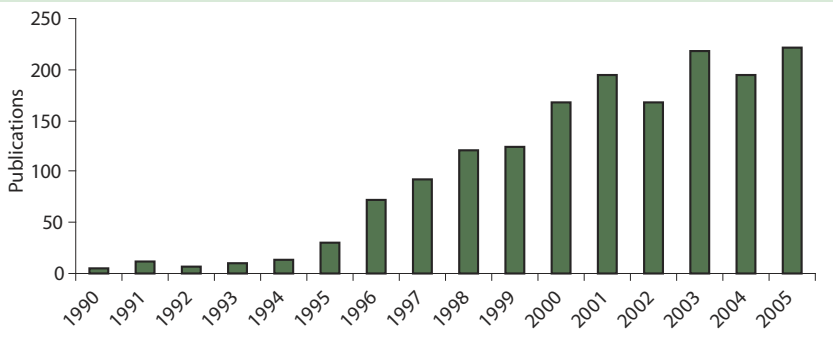


Table 1: *Pichia pastoris* expression system encompasses advantages of both prokaryotic (A–G) and eukaryotic (H) culture systems.

A	Chemically characterized media consisting of a basal media formulation complemented with trace elements, entirely protein free, avoiding concerns with animal-derived media components and offered at a fraction of the cost of mammalian cell culture media.
B	High growth rates from 0.04 to 0.1 μ per hour ($\mu = 1/x * dx/dt$, where x is concentration and t is time).
C	High expression levels due to its strong alcohol oxidase 1 (Aox1) promoter, which can make up as much as 35% of the soluble protein of the cell with typical expression yields in excess of 1 g/L. Expression levels are protein and fermentation protocol specific and have ranged from 20 mg/L to 14.8 g/L.
D	High cell densities in excess of 100 g/L dry cell weight.
E	Easy scalability due to robust nature of *P. pastoris*, which withstands large shear forces typical in large-scale industrial fermentors. *P. pastoris* has been scaled to 80,000-L fermentors.
F	Easy purification in case of secreted heterologous proteins; the purification process is simplified because *P. pastoris* does not secrete high amounts of endogenous protein.
G	Less expensive and easier to use than mammalian expression systems.
H	Mammalian-like proteins due to posttranslational modifications, including proteolytic processing, folding, disulfide bonds, and O- and N-linked glycosylation, are further enhanced by glycosylation engineering.

Common Host Strains

P. pastoris strains vary with respect to their ability to use methanol, leading to three principal phenotypes.

The **methanol utilization positive (Mut$^+$) phenotype** has functional Aox1 and Aox2 genes and grows on methanol at the wild-type rate. Its faster methanol growth rate compared with Aox-defective strains translates to faster production rates of heterologous protein. The **methanol utilization slow (Mut^S) phenotype** contains a disrupted Aox1 gene; its methanol metabolism depends solely on the transcriptionally weaker Aox2 gene, resulting in slower growth rates. The **methanol utilization negative (Mut$^-$)** phenotype has both Aox genes disrupted and, as a result, cannot metabolize methanol. Optimal protein expressions have been reported in all three phenotypes, so it is difficult to predict which phenotype will best express a protein of interest. Therefore, multiple expression vectors and host strains are often evaluated and screened.

Table 3 lists several of the common *P. pastoris* strains. The Y-11430 wild type is unsuitable for heterologous protein expression. GS115, the most

Table 2: Examples of *Pichia pastoris* expression levels (g/L) obtained in fermentors for various heterologous proteins of biopharmaceutical importance

Expressed Protein	Titer (g/L)	Reference
Vaccines		
Tetanus toxin fragment C	12	7
Heavy chain botulinum neurotoxin serotype A	1.72*	8
Antibody fragments		
A33scFv	4	9
Anti-HB Fab	0.05	10
Anti-HbsAg Fab	0.46	11
Hormones		
Human parathyroid hormone	0.3	12
Cytokines		
Human tumor necrosis factor	10	13
Bovine interferon gamma	1.0	14
Ovine interferon tau	0.4	15
Matrix proteins		
Mouse gelatin	14.8	16
Human collagen I–III	0.2–0.6	17

*Intracellular protein, expression level per gram of cell paste

Table 3: Common *Pichia pastoris* strains used for heterologous protein expression as they differ in genotype, phenotype, promoter, and induction

Strain	Genotype	Phenotype	Promoter	Induction
Y-11430	Wild-type	NRRL*	Aox1 and Aox2	Methanol
GS115	his4	Mut^+ His^-	Aox1 and Aox2	Methanol
KM71	Aox1::Sarg4 his4 arg4	Mut^S His^-	Aox1 disrupted	Methanol
MC100-3	Aox1::Sarg4 Aox2::Phis4 his4 arg4	Mut^- His^-	Aox1 and Aox2 disrupted	Glycerol and methanol
SMD1168	pep4 his4	Mut^+ His^- protease A deficient	Aox1 and Aox2	Methanol
SMD1165	prb1 his4	Mut^+ His^- protease B deficient	Aox1 and Aox2	Methanol

*Northern Regional Research Laboratories, Peoria, IL

commonly used expression host, has functional Aox1 and Aox2 genes and grows on methanol at the wild-type rate. The popularity of GS115 is based on its ability to grow to high cell densities in large-scale fermentors.

All unique His^- phenotypes were developed for selection purposes and are auxotrophic for histidine. They therefore require medium supplementation for growth unless His^+ expression cassettes are used. In the KM71 host, a derivative of GS115, the chromosomal Aox1 gene is largely deleted and replaced with the *S. cerevisiae* arg4 gene resulting in dependence on the much weaker Aox2 gene. The MC100-3 strain is deficient for both Aox1 and Aox2 and cannot grow on methanol.

Some secreted heterologous proteins are unstable in culture media where they are rapidly degraded by proteases. In high-density fermentation cultures, which typically contain small percentages of lysed cells, released proteases appear to be a significant factor in protein degradation. The use of host strains deficient in such proteases helps alleviate such enzymatic degradation. For example, the SMD1168 and SMD1165 strains are deficient, respectively, in protease A, which is encoded by pep4, and in protease B, which is encoded by prb1.

Fermentor Design and Operational Parameters

The use of *P. pastoris* for high cell density culture and protein expression requires careful evaluation of various fermentor design aspects and operating parameters as described in Table 4 (**28**, **33**). Photo 2 shows a typical system suitable for process development and small-scale production. Two critical design aspects are temperature control and oxygen supply.

Temperature Control: The heat production rates for glycerol and methanol substrates, respectively, are 2.6 and 4.5 kcal/g substrate consumed/hour. To remove excess heat, especially during the induction phase in high cell density cultures, autoclavable laboratory fermentors must have temperature control systems using jacketed culture vessels and closed-loop recirculation systems, with provisions to connect an external chiller when needed. For sterilizable-in-place fermentors, a dual heat-exchanger design with a connection to a chilled water or glycol loop is preferred.

Oxygen supply is achieved by proper blending of air and oxygen using either gas flow ratio control (GFRC) or conventional oxygen supplementation. GFRC allows the total gas flow rate to be fixed and the ratio between air and oxygen to vary depending on the oxygen demand of a culture. The oxygen consumption rates for glycerol and methanol substrates are respectively 13 and 35 mM O_2/g of substrate consumed/hour.

The common method of adding methanol using a septum and needle is not recommended because methanol will then pool and release in large droplets, leading to erratic dissolved oxygen (DO) profiles. That can easily be avoided by using a dip tube that is fully immersed in the culture. For the same reason, a variable-speed pump is recommended over fixed-speed pumps for dosing methanol because it does not need pulsing to achieve the required volumetric quantities.

Ammonium hydroxide is used to control pH and also serves as the nitrogen source. Ammonium hydroxide and methanol are volatile. They diffuse readily through silicone and other types of tubing, causing gaps of air. That can result in irregular methanol dosing leading to erratic DO profiles. We recommend the use of polytetrafluoroethylene (PTFE) or equivalent tubing throughout the process except for a small piece of chemically compatible flexible tubing to fit in the peristaltic pump head.

Inoculum Preparation and Growth Medium

The initial inoculum, 5–10% of the

initial culture volume, is typically prepared in baffled shake flasks containing minimal glycerol (MGY) or buffered minimal glycerol (BMGY) medium. MGY medium contains primarily a yeast nitrogen based (YNB) medium, typically 1% glycerol and small amounts of biotin. BMGY medium contains peptone and yeast extract, which may help stabilize secreted proteins.

A culture is initiated with *P. pastoris* cells derived from a frozen vial retrieved from the cell bank and is incubated in an orbital shaker at 30 °C, 250–300 rpm, for 16–24 hours until OD_{600} of 2–6 has been reached (**27**). This culture provides inoculum for the seed train, whereby each fermentor in turn inoculates the next larger reactor, providing a total of 5–10% of the fermentation volume in each step and representing scale-up factors in the range of 10–20 up to the production 80-m^3 fermentor.

The preferred fermentation growth medium consists of a basal salts (BS) medium containing 4% glycerol complemented with a trace element solution (**27**). The trace element solution is filter sterilized before use, whereas the BS medium is heat sterilized in the fermentor. The ability of *P. pastoris* to grow in simple, inexpensive, chemically characterized media without animal-derived growth factors or other troublesome media components (e.g., serum) alleviates some of today's regulatory concerns and makes it considerably more cost effective than complex chemically defined (CD) mammalian cell culture media.

Table 4: Fermentor design aspects and operational parameters for *Pichia pastoris* (vvm = volume of gas/volume of culture/min)

Inoculation	5–10% of Total Fermentor Volume
Agitation	Up to 1000 RPM for autoclavable lab-scale systems; requires Rushton impellers and four removable baffles to support high oxygen transfer rate.
Temperature	30 °C optimal; requires jacketed vessel for efficient heat removal using high cell density processes using Mut^+ strains and eventually additional chiller. Single wall vessel and cooling finger recommended only for low cell density of Mut^+ and for Mut^S and Mut^- strains only. Growth above 32 °C is detrimental to protein expression.
Gassing rate	1–1.25 vvm total gas flow; requires multiple large-orifice ring sparger. Higher gas flow rates are not recommended due to increased methanol evaporation.
Gas Mix	Air and oxygen; through either gas flow ratio control using one mass flow controller per gas or oxygen supplementation control with one mass flow meter for total gas flow
Dissolved oxygen	35% air saturation; requires sequential cascade control programming to agitation, gas mix, and substrate.
pH	5.0 ± 0.1 in glycerol batch and fed-batch phase, 2–5 in methanol induction phase depending on protein stability
Substrate 1	Glycerol 50%, feed rate 15 mL/hr/L culture volume; requires fixed or variable speed peristaltic pump for volumetric feed, and optional weighing scale for gravimetric feed, or pressure addition system.
Substrate 2	Methanol 100%, feed rate 1–12 mL/hr/L culture volume for Mut^+ and 1–6 mL/hr/L culture volume for Mut^S strains; requires variable speed peristaltic pump with PTFE tubing or equivalent for volumetric feed, optional weighing scale for gravimetric feed, or pressure addition system. Fully immersed dip tube in culture medium is required.
Methanol	0.4–4% for Mut^+ and Mut^S strains, 0.2-0.8% for Mut^- strains and 0.5% for Mut^-. On-line methanol sensor or off-line analytical analyzer is recommended.
Antifoam	5% Struktol JA 673 in 100% methanol, KF0673 Kabo Jackson or equivalent; requires fixed-speed peristaltic pump.
Base	30% NH_4OH; requires fixed-speed peristaltic pump and PTFE tubing or equivalent.

Fermentation Protocol and Control Strategies

The standard culture protocol is to grow *P. pastoris* in excess glycerol to repress protein expression, followed by methanol induction to initiate heterologous protein expression as the glycerol becomes exhausted. Figure 2 shows a schematic lay-out for a typical fermentor set-up. Protein expression with all three phenotypes is performed using a culture strategy in three phases (Figure 3). Note that the first two phases are the same for all phenotype expression hosts, with phase III depending on the specific host phenotype. Sampling is typically performed twice daily for analysis of cell growth at OD_{600} and recombinant protein concentration or activity during phase III.

Phase I is the glycerol batch culture to create biomass. A maximum initial concentration of 4% glycerol is recommended because of cellular toxicity associated with higher levels. The initial culture volume is 65–75% or 45–60% of the total reactor volume for induction phases of less or more than 40 hours, respectively. DO is typically controlled through a sequential control cascade programmed first to agitation and subsequently to either GFRC or oxygen supplementation, depending on the preferred air and oxygen gas blending strategy. GFRC gas blending is often preferred because it not only maintains a constant total gas flow, but also monitors and controls both air and oxygen individually through mass flow controllers.

Alternatively, a more conventional oxygen supplementation gas mixing strategy can be used with one mass flow controller for total gas flow. For most installations, the agitation range set in the DO cascade controller is typically between 30% of minimum and maximum rpm. At the end of the batch phase, when the initial amount of glycerol in the fermentor is completely exhausted, oxygen demand will sharply decline. Setting the DO controller to "off" will result in a sharp rise of the DO process value (DO spike). But with the DO controller active, the DO spike will be masked by a rapid decline of the amount of oxygen in the gas mixture, which is an essential attribute of modern PID (proportional-integral-derivative) controllers**.** Nevertheless, the oxygen decline can be readily monitored in the GFRC configuration. A cellular yield of 90–150 g/L wet cells is expected at the end of phase I.

Phase II is the glycerol fed-batch culture, which limits glycerol, thereby derepressing the methanol metabolic pathways and allowing cells to

Photo 2: Biostat B Plus laboratory fermentorin a twin configuration with gas flow ratio control for air and oxygen suitable for *Pichia pastoris* process development and small-scale production. SARTORIUS BBI SYSTEMS, BETHLEHEM, PA (SARTORIUS-BBI-SYSTEMS.COM)

Table 5: Selected physical properties of methanol

Boiling point	64.7 °C
Flash point, closed cup	12.2 °C
Flash point, open cup	15.6 °C
Explosion limits, vol% in air	5.5–44
Relative density to water	0.7915
Solubility in water	Miscible
Vapor pressure at 20 °C	12.3 kPa
Relative vapor density to air	1.1

transition smoothly from glycerol to methanol growth (**34**). Because it is critical that DO and oxygen flow rate are properly balanced in phase II, the DO cascade control should be changed only at the end of phase I either after a DO spike or in its absence if the oxygen flow rate has sufficiency declined. At this point the DO cascade can be changed to substrate (glycerol) supplementation at a growth-limiting rate, while new fixed set points for both air and oxygen flow rates and gas ratios ensure that oxygen is in abundant supply. The total gas flow remains at 1–1.25 vvm. The agitation speed is typically set between 80 to 100% of maximum rpm.

DO should never be allowed to drop below 20% of air saturation; therefore, appropriate alarm thresholds should be programmed in the controller. Or to ensure growth-limiting feed of glycerol, feed pumps can be shut off manually and the time to allow DO to rise by 10% measured. If the lag time for the DO spike is short, generally less than one minute, glycerol is assumed to be limiting. Once DO has raised by 10%, the glycerol feed pump can be turned on again. If a DO cascade control for glycerol is unavailable, it is also possible to add glycerol at a fixed rate of typically 15 mL/hr/L of culture volume. As long as glycerol is added at a fixed rate, the rate of oxygen use will also be fixed, allowing DO control through either GFRC or oxygen supplementation as described earlier, with the exception of deleting agitation from the control cascade. At the end of phase II, a cellular yield of 180–220 g/L wet cells should be achieved.

Phase III is the methanol fed-batch culture or induction phase, which differs according to each expression host phenotype. In all cases, glycerol must be exhausted before methanol feed starts. This can be accomplished by turning off the glycerol pump and DO cascade control allowing the DO to rise from 30% to 100% (DO spike). Note that in the case of a Mut^+ host strain it may take one to three hours for DO to stabilize back at 30%. If the DO cascade remains active, the amount of oxygen in the blend mixture will decrease over time. To ensure that methanol does not accumulate during the induction phase, frequent DO spikes can be performed manually if needed. The DO cascade control strategies used in phase II with air and oxygen gas blending through either GFRC or oxygen supplementation can still be used, but it is quite common to maintain fixed set points for air and oxygen gas flow rate and blend ratio to ensure that oxygen is in abundant supply and methanol will be added at a growth limiting rate. It is also possible to program a cascade to substrate (methanol). In any case, methanol levels in the fermentor are critical and must be carefully controlled. At the end of the induction phase, a final cellular yield of 350–450 g/L of wet cells can be obtained.

The most common induction protocol is to use a progressive methanol dosing profile. For Mut^+ strains this feed rate is typically initiated at 3.5 mL/hr/L of culture volume. Once a culture adapts to its current level of methanol, which may take two to five hours, the feed rate is increased (using either a preprogrammed dosing profile, or manually by adjusting set points) in increments of 1 mL/hr/L of culture volume every one to two hours until achieving a feed rate of 11 to 12 mL/hr/L. To maximize protein expression levels, the methanol feed rate should be brought to maximum as quickly as possible, but optimum induction time profiles can be derived only through analysis of time-based samples (**35**). For many recombinant proteins, a direct correlation between the amounts of methanol consumed and protein produced has been observed (**36**). That observation can be extrapolated to the consumption of other carbon sources as well as to cases of mixed feed strategies.

In recent years sensor technology has become available to directly measure methanol in situ, thereby preventing starvation or intoxication of cultures (**9, 37, 38**). In this way a specific methanol concentration of 0.4–4.0% can be maintained in culture using a feedback control loop with a methanol substrate pump. Mut^+ strains are very sensitive to higher methanol concentrations, and if the DO drops below 15% due to an excess of methanol, the methanol feed pump must be turned off to allow the culture to metabolize it. An intuitive operator response would be to increase the oxygen flow rate, but a sudden increase in oxygen in the presence of an excess amount of methanol (1–2%) can result in accumulation of toxic levels of formaldehyde (the first product of methanol metabolism in the peroxisomes), leading to cell death (**21**).

The induction protocol for the Mut^S strains is the same as for the Mut^+ strains except for a lower methanol feed rate. That rate is initiated at 1 mL/hr/L of culture volume and subsequently increased in 10% increments every 30 minutes to a maximum of 3 mL/hr/L of culture volume. The methanol concentration must remain within 0.2–0.8% for Mut^S strains (**39, 40**). DO spikes cannot be used effectively to evaluate cultures because oxygen consumption is too slow with methanol as the sole carbon source. An alternative induction strategy for Mut^S strains is to use a mixed methanol/glycerol (delivered in g/hr) feed, e.g., in ratios

Figure 2: Schematic layout of a *P. pastoris* fermentation system. The fermentor is equipped with a powerful motor (1), exhaust condenser (2), foam probe (3), multiple Rushton impellers (4), a multiorifice ring sparger (5), and sensors for temperature, pH, DO, and methanol (6). Air and oxygen are added through individual solenoid valves (7) and mass flow controllers (8). The liquid substrates methanol (MeOH) and glycerol as well as base (NH_4OH) and antifoam (AF) are added through peristaltic pumps (9). Glycerol and methanol substrate addition bottles and/or the entire culture vessel are put on weighing scales (10). Fixed lines (—): both actual soft or hard piping; dotted lines (----): electrical wiring from actuators and sensors to the controller.

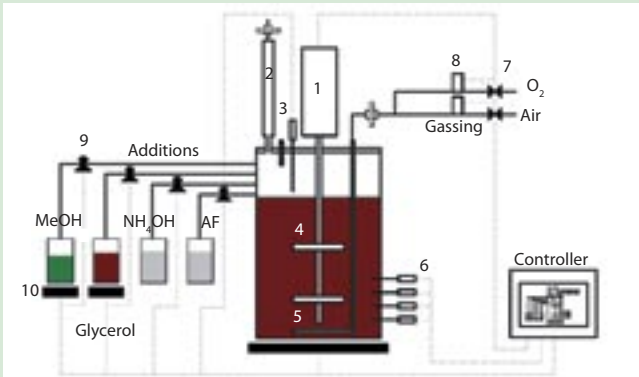
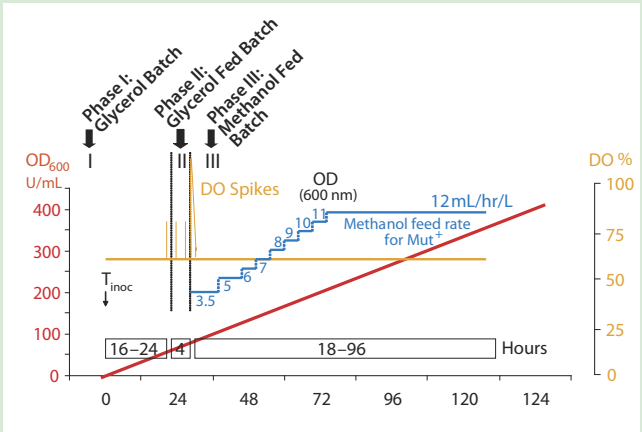


Figure 3: Model growth curve of *Pichia pastoris* Mut+ host strain in optical density (OD) units/mL at 600 nm (left) and dissolved oxygen (DO) set point in % air saturation (right) over the time course of the fermentation in hours; graph features three distinct culture phases: glycerol batch (16–24 hours), glycerol fed-batch (four hours), and methanol fed batch (18–96 hours) along with a typical methanol feed profile in mL/hr per L culture volume for a Mut+ host strain. The DO value is basically kept constant at 35% except for multiple DO spikes to evaluate the state of the culture.



ranging from 4/1 to 1/4 (**34**). Some empirically determined protocols achieve the same effect by gradually changing the proportion of methanol in a feedstream. Because mixed feed strategies reduce the overall cooling and oxygen requirements for fermentation, this feature may become of particular interest in evaluating fermentor design.

Mut⁻ strains require an alternative carbon source — glycerol or sorbitol, for example (**41**). Despite the fact that methanol cannot be metabolized by Mut⁻ strains, its presence will still induce transcription of the Aox1 promoter and expression of genes regulated by it (**42**). However, excess glycerol can repress the Aox1 gene, and the induction strategy for Mut⁻ strains follows the mixed feeding strategy for Mut^S strains. Glycerol is fed at 1 g/hr/L of culture volume under growth-limiting conditions, and methanol is maintained at 0.5% during the induction phase (**40**). An on-line methanol sensor is required here because DO spikes cannot effectively measure methanol limitation.

Facility Considerations

The most important facility consideration that arises when using *P. pastoris* involves the safe use of methanol. A clear, volatile, colorless, toxic, flammable liquid with a faint odor like alcohol, methanol can pose a serious fire hazard. It mixes well with air, easily forming explosive mixtures. Table 5 lists its important physical properties.

The typical methanol concentrations in fermentation broth are too low to raise any particular safety concerns, leaving the actual transport, handling, and storage of large amounts as the predominant hazard. Because methanol is widely used as an industrial and laboratory solvent, many safety recommendations and best practices have originated in the chemical industry. Methanol should be stored in clean containers made from carbon steel, stainless steel, high-density polyethylene (HDPE), or vulcanized natural rubber. Storage containers should be constructed with an internal floating roof and an inert gas pad to minimize vapor emissions. Compressed air should never be used for filling, discharging, or handling. Inert gas (e.g., nitrogen) can be used, but pumping is generally preferred. Standard preventive measures to alleviate explosion risk are the use of closed fermentation systems, ventilation and explosion-proof electrical equipment and lighting, and nonsparking hand tools. The explosion risk can also be addressed by using well-mixed methanol–water feeds with increased the flash points. The principal disadvantage of that would be increased feed volumes during fermentation.

For further information please consult international, federal, state, and local regulations, best practice guidelines from industry organizations, and in-house occupational health and safety programs (**43–45**).

A Cost-Effective Host

Significant improvements in glycosylation engineering have further propelled *P. pastoris* as a cost-effective expression host for the manufacture of various vaccines and biopharmaceuticals, including biogenerics. Understanding and applying the molecular biology and protein chemistry foundations to select expression vectors and hosts is prerequisite to successful implementation of this fermentation strategy, which in itself cannot be developed without a careful evaluation of the design and operational parameters of fermentor systems. Critical design considerations are temperature control, oxygen requirements, and methanol feeding algorithms, along with facility considerations associated with the use of large quantities of methanol.

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