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Pichia Fermentation Process Guidelines

Overview

Introduction

Pichia pastoris, like *Saccharomyces cerevisiae*, is particularly well-suited for fermentative growth. *Pichia* has the ability to reach very high cell densities during fermentation which may improve overall protein yields.

We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Since there are a wide variety of fermenters available, it is difficult to provide exact procedures for your particular case. The guidelines given below are based on fermentations of both Mut^+ and Mut^S *Pichia* strains in a 15 liter table-top glass fermenter. Please read the operator's manual for your particular fermenter before beginning. The table below provides an overview of the material covered in these guidelines.

Fermentation Parameters

It is important to monitor and control the following parameters throughout the fermentation process. The following table describes the parameters and the reasons for monitoring them.

Parameter	Reason
Temperature (30.0°C)	Growth above 32°C is detrimental to protein expression
Dissolved oxygen (>20%)	*Pichia* needs oxygen to metabolize glycerol and methanol
pH (5.0-6.0 and 3.0)	Important when secreting protein into the medium and for optimal growth
Agitation (500 to 1500 rpm)	Maximizes oxygen concentration in the medium
Aeration (0.1 to 1.0 vvm* for glass fermenters)	Maximizes oxygen concentration in the medium which depends on the vessel
Antifoam (the minimum needed to eliminate foam)	Excess foam may cause denaturation of your secreted protein and it also reduces headspace
Carbon source (variable rate)	Must be able to add different carbon sources at different rates during the course of fermentation

* volume of oxygen (liters) per volume of fermentation culture (liters) per minute

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Overview, continued

Recommended Equipment

Below is a checklist for equipment recommendations.

- A jacketed vessel is needed for cooling the yeast during fermentation, especially during methanol induction. You will need a constant source of cold water (5-10°C). This requirement may mean that you need a refrigeration unit to keep the water cold.
- A foam probe is highly recommended as antifoam is required.
- A source of O_2--either air (stainless steel fermenters at 1-2 vvm) or pure O_2 (0.1-0.3 vvm for glass fermenters).
- Calibrated peristaltic pumps to feed the glycerol and methanol.
- Automatic control of pH.

Medium Preparation

You will need to prepare the appropriate amount of following solutions:

- Fermentation Basal Salts (page 11)
- PTM_1 Trace Salts (page 11)
- ~75 ml per liter initial fermentation volume of 50% glycerol containing 12 ml PTM_1 Trace Salts per liter of glycerol.
- ~740 ml per liter initial fermentation volume of 100% methanol containing 12 ml PTM_1 Trace Salts per liter of methanol.

Monitoring the Growth of *Pichia pastoris*

Cell growth is monitored at various time points by using the absorbance at 600 nm (OD_{600}) and the wet cell weight. The metabolic rate of the culture is monitored by observing changes in the concentration of dissolved oxygen in response to carbon availability (see next page).

Dissolved Oxygen (DO) Measurement

Introduction

The dissolved oxygen concentration is the relative percent of oxygen in the medium where 100% is O_2-saturated medium. *Pichia* will consume oxygen as it grows, reducing the dissolved oxygen content. However, because oxygen is required for the first step of methanol catabolism, it is important to maintain the dissolved oxygen (DO) concentration at a certain level (>20%) to ensure growth of *Pichia* on methanol. Accurate measurement and observation of the dissolved oxygen concentration of a culture will give you important information about the state and health of the culture. Therefore, it is important to accurately calibrate your equipment. Please refer to your operator's manual.

Maintaining the Dissolved Oxygen Concentration (DO)

1. Maintaining the dissolved oxygen above 20% may be difficult depending on the oxygen transfer rates (OTR) of the fermenter, especially in small-scale glass vessels. In a glass vessel, oxygen is needed to keep the DO above 20%, usually ~0.1-0.3 vvm (liters of O_2 per liter of fermentation culture per minute). Oxygen consumption varies and depends on the amount of methanol added and the protein being expressed.
2. Oxygen can be used at 0.1 to 0.3 vvm to achieve adequate levels. This can be accomplished by mixing with the air feed and can be done in any glass fermenter. For stainless steel vessels, pressure can be used to increase the OTR. Be sure to read the operator's manual for your particular fermenter.
3. If a fermenter cannot supply the necessary levels of oxygen, then the methanol feed should be scaled back accordingly. Note that decreasing the amount of methanol may reduce the level of protein expression.
4. To reach maximum expression levels, the fermentation time can be increased to deliver similar levels of methanol at the lower feed rate. For many recombinant proteins, a direct correlation between amount of methanol consumed and the amount of protein produced has been observed.

Use of DO Measurements

During growth, the culture consumes oxygen, keeping the DO concentration low. Note that oxygen is consumed whether the culture is grown on glycerol or methanol. The DO concentration can be manipulated to evaluate the metabolic rate of the culture and whether the carbon source is limiting. The metabolic rate indicates how healthy the culture is. Determining whether the carbon source is limiting is important if you wish to fully induce the *AOX1* promoter. For example, changes in the DO concentrations (DO spikes) allow you to determine whether all the glycerol is consumed from the culture before adding methanol. Secondly, it ensures that your methanol feed does not exceed the rate of consumption. Excess methanol (> 1-2% v/v) may be toxic.

Manipulation of DO

If carbon is limiting, shutting off the carbon source should cause the culture to decrease its metabolic rate, and the DO to rise (spike). Terminate the carbon feed and time how long it takes for the DO to rise 10%, after which the carbon feed is turned back on. If the lag time is short (< 1 minute), the carbon source is limiting.

Fermenter Preparation and Glycerol Batch Phase

Inoculum Seed Flask Preparation

Remember not to put too much medium in the baffled flasks. Volume should be 10-30% of the total flask volume.

1. Baffled flasks containing a total of 5-10% of the initial fermentation volume of MGY or BMGY are inoculated with a colony from a MD or MGY plate or from a frozen glycerol stock.
2. Flasks are grown at 30°C, 250-300 rpm, 16-24 hours until OD_{600} = 2-6. To accurately measure OD_{600} > 1.0, dilute a sample of your culture 10-fold before reading.

Glycerol Batch Phase

1. Sterilize the fermenter with the Fermentation Basal Salts medium containing 4% glycerol (see page 11).
2. After sterilization and cooling, set temperature to 30°C, agitation and aeration to operating conditions (usually maximum rpm and 0.1-1.0 vvm air), and adjust the pH of the Fermentation Basal Salts medium to 5.0 with 28% ammonium hydroxide (undiluted ammonium hydroxide). Add aseptically 4.35 ml PTM_1 trace salts/liter of Fermentation Basal Salts medium.
3. Inoculate fermenter with approximately 5-10% initial fermentation volume from the culture generated in the inoculum shake flasks. Note that the DO will be close to 100% before the culture starts to grow. As the culture grows, it will consume oxygen, causing the DO to decrease. Be sure to keep the DO above 20% by adding oxygen as needed.
4. Grow the batch culture until the glycerol is completely consumed (18 to 24 hours). This is indicated by an increase in the DO to 100%. Note that the length of time needed to consume all the glycerol will vary with the density of the initial inoculum.
5. Sampling is performed at the end of each fermentation stage and at least twice daily. We take 10 ml samples for each time point, then take 1 ml aliquots from this 10 ml sample. Samples are analyzed for cell growth (OD_{600} and wet cell weight), pH, microscopic purity, and protein concentrations or activity. Freeze the cell pellets and supernatants at -80°C for later analysis. Proceed to **Glycerol Fed-Batch Phase**, page 5.

Yield

A cellular yield of 90 to 150 g/liter wet cells is expected for this stage. Recombinant protein will not yet be produced due to the absence of methanol.

Glycerol Fed-Batch Phase

Introduction

Once all the glycerol is consumed from the batch growth phase, a glycerol feed is initiated to increase the cell biomass under limiting conditions. When you are ready to induce with methanol, you can use DO spikes to make sure the glycerol is limited.

Glycerol Fed-Batch Phase

1. Initiate a 50% w/v glycerol feed containing 12 ml PTM_1 trace salts per liter of glycerol feed. Set the feed rate to 18.15 ml/hr /liter initial fermentation volume.
2. Glycerol feeding is carried out for about four hours or longer (see below). A cellular yield of 180 to 220 g/liter wet cells should be achieved at the end of this stage while no appreciable recombinant protein is produced.

Note

The level of expressed protein depends on the cell mass generated during the glycerol fed-batch phase. The length of this feed can be varied to optimize protein yield. A range of 50 to 300 g/liter wet cells is recommended for study. A maximum level of 4% glycerol is recommended in the batch phase due to toxicity problems with higher levels of glycerol.

Important

If dissolved oxygen falls below 20%, the glycerol or methanol feed should be stopped and nothing should be done to increase oxygen rates until the dissolved oxygen spikes. At this point, adjustments can be made to agitation, aeration, pressure or oxygen feeding.

Proteases

In the literature, it has been reported that if the pH of the fermentation medium is lowered to 3.0, neutral proteases are inhibited. If you think neutral proteases are decreasing your protein yield, change the pH control set point to 3.0 during the glycerol fed-batch phase (above) or at the beginning of the methanol induction (next page) and allow the metabolic activity of the culture to slowly lower the pH to 3.0 over 4 to 5 hours (Brierley, *et al.*, 1994; Siegel, *et al.*, 1990).

Alternatively, if your protein is sensitive to low pH, it has been reported that inclusion of casamino acids also decreases protease activity (Clare, *et al.*, 1991).

Methanol Fed-Batch Phase

Introduction

All of the glycerol needs to be consumed before starting the methanol feed to fully induce the *AOX1* promoter on methanol. However, it has been reported that a "mixed feed" of glycerol and methanol has been successful to express recombinant proteins (Brierley, *et al.*, 1990; Sreekrishna, *et al.*, 1989). It is important to introduce methanol slowly to adapt the culture to growth on methanol. If methanol is added too fast, it will kill the cells. Once the culture is adapted to methanol, it is very important to use DO spikes to analyze the state of the culture and to take time points over the course of methanol induction to optimize protein expression. Growth on methanol also generates a lot of heat, so temperature control at this stage is very important.

Mut$^+$ Methanol Fed-Batch Phase

1. Terminate glycerol feed and initiate induction by starting a 100% methanol feed containing 12 ml PTM_1 trace salts per liter of methanol. Set the feed rate to 3.6 ml/hr per liter initial fermentation volume.
2. During the first 2-3 hours, methanol will accumulate in the fermenter and the dissolved oxygen values will be erratic while the culture adapts to methanol. Eventually the DO reading will stabilize and remain constant.
3. If the DO cannot be maintained above 20%, stop the methanol feed, wait for the DO to spike and continue on with the current methanol feed rate. Increase agitation, aeration, pressure or oxygen feeding to maintain the DO above 20%.
4. When the culture is fully adapted to methanol utilization (2-4 hours), and is limited on methanol, it will have a steady DO reading and a fast DO spike time (generally under 1 minute). Maintain the lower methanol feed rate under limited conditions for at least 1 hour after adaptation before doubling the feed. The feed rate is then doubled to ~7.3 ml/hr/liter initial fermentation volume.
5. After 2 hours at the 7.3 ml/hr/liter feed rate, increase the methanol feed rate to ~10.9 ml/hr per liter initial fermentation volume. This feed rate is maintained throughout the remainder of the fermentation.
6. The entire methanol fed-batch phase lasts approximately 70 hours with a total of approximately 740 ml methanol fed per liter of initial volume. However, this may vary for different proteins.

Note: The supernatant may appear greenish. This is normal.

Yield

The cell density can increase during the methanol fed-batch phase to a final level of 350 to 450 g/liter wet cells. Remember that because most of the fermentation is carried out in a fed-batch mode, the final fermentation volume will be approximately double the initial fermentation volume.

Fermentation of Mut^S *Pichia* Strains

Since Mut^S cultures metabolize methanol poorly, their oxygen consumption is very low. Therefore, you cannot use DO spikes to evaluate the culture. In standard fermentations of a Mut^S strain, the methanol feed rate is adjusted to maintain an excess of methanol in the medium which does not exceed 0.3% (may be determined by gas chromatography). While analysis by gas chromatography will insure that nontoxic levels of methanol are maintained, we have used the empirical guidelines below to express protein in Mut^S strains. A gas chromatograph is useful for analyzing and optimizing growth of Mut^S recombinants.

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Methanol Fed-Batch Phase, continued

Mut^S Methanol Fed- Batch Phase

The first two phases of the glycerol batch and fed-batch fermentations of the Mut^S strains are conducted as described for the Mut^+ strain fermentations. The methanol induction phases of the Mut^+ and Mut^S differ in terms of the manner and amount in which the methanol feed is added to the cultures.

1. The methanol feed containing 12 ml PTM_1 trace salts per liter of methanol is initiated at 1 ml/hr/liter initial fermentation volume for the first two hours. It is then increased in 10% increments every 30 minutes to a rate of 3 ml/hr which is maintained for the duration of the fermentation.
2.. The vessel is then harvested after ~100 hours on methanol. This time may be varied to optimize protein expression.

Harvesting and Lysis of Cells

Introduction

The methods and equipment listed below are by no means complete. The amount of cells or the volume of supernatant will determine what sort of equipment you need.

Harvesting Cells and Supernatant

For small fermentations (1-10 liters), the culture can be collected into centrifuge bottles (500-1000 ml) and centrifuged to separate the cells from the supernatant.

For large fermentations, large membrane filtration units (Millipore) or a Sharples centrifuge can be used to separate cells from the supernatant. The optimal method will depend on whether you need the cells or the supernatant as the source of your protein and what you have available.

Supernatants can be loaded directly onto certain purification columns or concentrated using ultrafiltration.

Cell Lysis

We recommend cell disruption using glass beads as described in *Current Protocols in Molecular Biology*, page 13.13.4. (Ausubel, *et al.*, 1990) or *Guide to Protein Purification* (Deutscher, 1990). This method may be tedious for large amounts of cells. For larger amounts, we have found that a microfluidizer works very well. French pressing the cells does not seem to work as well as the glass beads or the microfluidizer.

References

Introduction

Most of the references below refer to papers where fermentation of *Pichia* was performed. Note that some of these are patent papers. You can obtain copies of patents using any of the following methods.

- **Patent Depository Libraries.** U. S. patents and international patents granted under the Patent Cooperation Treaty (PCT) are available on microfilm. These can be copied and mailed or faxed depending on length. There is a fee for this service. The reference librarian at your local library can tell you the location of the nearest Patent Depository Library.
- **Interlibrary Loan.** If you are not near a Patent Depository Library, you may request a copy of the patent through interlibrary loan. There will be a fee for this service.
- **U. S. Patent Office.** Requests may be made directly to the Patent Office, Arlington, VA. Please call 703-557-4636 for more information on cost and delivery.
- **Private Library Services.** There are private companies who will retrieve and send you patents for a fee. Two are listed below:

  Library Connection: 804-758-3311
  Rapid Patent Services: 800-336-5010

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Recipes

Fermentation Basal Salts Medium

For 1 liter, mix together the following ingredients:

Phosphoric acid, 85%	(26.7 ml)
Calcium sulfate	0.93 g
Potassium sulfate	18.2 g
Magnesium sulfate-7H_2O	14.9 g
Potassium hydroxide	4.13 g
Glycerol	40.0 g
Water	to 1 liter

Add to fermenter with water to the appropriate volume and sterilize.

PTM_1 Trace Salts

Mix together the following ingredients:

Cupric sulfate-5H_2O	6.0 g
Sodium iodide	0.08 g
Manganese sulfate-H_2O	3.0 g
Sodium molybdate-2H_2O	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-7H_2O	65.0 g
Biotin	0.2 g
Sulfuric Acid	5.0 ml
Water	to a final volume of 1 liter

Filter sterilize and store at room temperature.

Note: There may be a cloudy precipitate upon mixing of these ingredients. Filter-sterilize as above and use.