Objectives

At the end of this lab, students will be able to

- compare the effects of different carbon sources on transcription of genes controlled by the yeast \( \text{GAL1} \) promoter.

- explain the roles of heat and detergents in the preparation of yeast cell extracts.

- culture yeast with different carbon sources to induce or repress expression from the \( \text{GAL1} \) promoter.

- prepare extracts from transformed yeast strains suitable for use in SDS-PAGE gels and western blots.
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Over the next few weeks, you will be analyzing the expression of *S. pombe* and *S. cerevisiae* Met or bacterial LacZ fusion proteins in your transformed strains. You have already tested the ability of the overexpression plasmids to complement the *met* mutation in your yeast strains. Complementation depends on the presence of functional Met proteins. If you observed a failure to complement the *met* deficiency, this could indicate that proteins were not expressed from the plasmids. Alternatively, the overexpressed proteins may be present, but not function normally. Remember that the proteins expressed from the BG1805 and pYES2.1 plasmids are fusion proteins with additional sequences at their C-termini (Gelperin *et al.*, 2005). The biochemical activities of these fusion proteins have not been previously evaluated. (We are the first to test whether the Met fusion proteins function similarly to the normal *S. cerevisiae* proteins!)

In this experiment, you will prepare extracts from yeast for later experiments (Chapters 14 and 15) in which you will determine if the Met and LacZ fusion proteins are being successfully expressed in the transformed yeast strains and how the expression of the fusion proteins varies with carbon sources.

Regulation of the *GAL1* promoter

In yeast, glycolysis plays a major role in energy production, and glucose is far and away its preferred carbon source. Genes involved in the metabolism of other carbon sources are usually repressed when glucose is available. When glucose is not available, however, yeast activate genes that metabolize other available energy sources, such as galactose. Galactose increases the transcription of several genes for enzymes that transport galactose into cells and ultimately convert it into glucose-6-phosphate (G6P), an intermediate in glycolysis. The first gene in the pathway induced by galactose, *GAL1*, encodes galactokinase, which phosphorylates galactose to galactose-1-phosphate. (Check out the *GAL1* pathways link in SGD.) The *GAL1* promoter has been incorporated upstream of the ORF site in both the pBG1805 and pYES2.1 plasmids and therefore controls transcription of plasmid-encoded *MET*/Met and *lacZ* genes in transformed cells.

The figure on the opposite page provides a simple overview of gene expression from the *GAL1* promoter in the presence of glucose, raffinose and galactose. The promoter contains both negative and positive regulatory sites encoded within its DNA sequence. In the presence of glucose, repressor proteins bind to the negative regulatory sites and repress transcription. The Gal4p transcriptional activator binds to positive regulatory sites. Gal4p is a transcription factor that binds to DNA as a dimer. (The figure at the beginning of this chapter shows the crystal structure of the DNA binding and dimerization domains of Gal4p complexed with DNA.) In the presence of glucose, Gal4p is inactive, because it is bound to the repressor protein, Gal80p.

Glucose repression can be relieved by growing cells in a poor carbon source, such as raffinose. Raffinose is a trisaccharide composed of galactose, fructose and glucose. Raffinose is not able to induce high levels of *GAL1* expression, which requires galactose. In the presence of galactose, expression of the *GAL1* gene increases ~1000-fold above the level observed in the
Protein overexpression

Regulation of the \textit{GAL1} promoter. In the presence of glucose, transcription is repressed because repressor proteins bind to regulatory sites in the DNA and to the Gal4p transcriptional activator. Glucose repression is relieved in the presence of raffinose, but Gal4p remains inactive. Gal4p activates transcription in the presence of galactose due to the removal of the Gal80p protein.

In the presence of glucose, this stimulation is primarily due to the activity of Gal4p, which is no longer bound to the inhibitory Gal80p protein. Gal4p acts as a master regulator of galactose metabolism. In addition to activating \textit{GAL1} transcription, Gal4p also binds to the promoters of the \textit{GAL7} and \textit{GAL10} genes, which are situated adjacent to the \textit{GAL1} gene on yeast chromosome 2. Like \textit{GAL1}, the \textit{GAL7} and \textit{GAL10} genes encode proteins involved in galactose metabolism.

Preparing protein extracts from yeast cells

Proteins comprise about half of the dry weight of most cells and include the many structural proteins, catalysts, receptors and signaling proteins responsible for cell function. To understand cell function, scientists often want to analyze the protein composition of cells. Protein analysis begins with the preparation of a cell extract, ideally under conditions that minimize protein degradation. Preparing good cell extracts is something of an art, and many factors need to be considered during the design of an extraction protocol.

In this course, we will be analyzing protein function in yeast. An average haploid yeast cell contains \texttildelow ~6 pg protein (Sherman, 2002). Although yeast cells have many advantages for genetic studies, they are notoriously difficult to use for biochemical studies. Nonetheless, scientists have been able to develop procedures for extracting yeast proteins that circumvent these experimental barriers.
The first consideration in designing an extraction procedure is the compartmentalization of cells. All cells are surrounded by a plasma membrane and eukaryotic cells contain additional membranes that surround organelles. Fungal cells also have cellulose-based cell walls that protect the cells against mechanical and osmotic forces. Cell extraction procedures begin with the disruption of the plasma membrane and cell wall by mechanical and/or chemical treatments. Mechanical disruption of yeast cells must be fairly vigorous because their cell walls are very tough. Mechanical methods commonly used to disrupt yeast include sonication, high pressure, and “beating” with glass beads. These vigorous treatments run the risk of damaging proteins because of the heat and foaming generated during the processes.

Chemical treatments offer a gentler alternative to mechanical disruption for preparing extracts. Chemical extraction procedures frequently include detergents that solubilize membrane lipids, thereby allowing proteins to diffuse out of the cell. Most detergents do not discriminate between intracellular and plasma membranes, so a detergent extract usually contains proteins from multiple organelles as well as cytoplasmic proteins. In this experiment, we will use sodium dodecyl sulfate (SDS) as the detergent. SDS is a denaturing detergent that unfolds protein structures by breaking the thousands of weak bonds that normally stabilize protein structures. The proteins are converted to random coils coated along their lengths by negatively charged SDS molecules.

When preparing extracts, care must be taken to protect proteins from degradation by cellular proteases. Cells contain proteases with many different specificities that are responsible for normal turnover of proteins in cells. Cell disruption often releases proteases from compartments such as lysosomes, providing them access to cytoplasmic proteins. Yeast are notoriously rich in proteases. In an intact yeast cell, many of these proteases are located in the yeast vacuole, which is analogous to the mammalian lysosome. The protocol that we will use in this course (Amberg et al., 2005) uses a combination of heat and SDS to rapidly destroy the yeast proteases. Cell suspensions are immediately plunged into a boiling water bath after the addition of SDS. Extracts prepared by this method are suitable for electrophoresis and western blot analysis.
Exercise 1 - Prepare cell cultures for extraction

DAY 1

1. Each student should collect two culture tubes containing 1 mL of YC-Ura medium with 2% raffinose. Each of two tubes should have a **DIFFERENT** color cap. The different colored caps are used to indicate whether the culture will be given glucose or galactose on Day 2 of the experiment. Label each tube with the name of your strain and the plasmid transformed into it.

2. Inoculate each tube with a single yeast colony. Together, each team will prepare 6 cultures, including 2 cultures of each transformed strain. The two samples of each culture should be given caps of different colors.

3. Place the cultures in the 30˚C shaking incubator.

DAY 2 - Four to five hours before your lab class, the teaching staff will:

- add 1 mL of YC+glucose (repression medium) to the raffinose cultures with one color cap.
- add 1 mL YC + galactose (induction medium) to raffinose cultures with the other color cap.

You will be told which color corresponds to which sugar source

Exercise 2 - Preparing cell extracts

Prepare the cells for extraction

1. Collect your team's 6 cultures and note which cultures contain glucose and which cultures contain galactose.

2. Determine the cell concentration or each culture. Vortex the tubes and transfer 100 µL of each cell culture to 900 µL deionized water. Measure the OD$_{600}$ of cultures in the spectrophotometer. Note the values in your notebook. You will refer to them later when interpreting your SDS-PAGE gels (Chapter 14).

3. Transfer 1.5 mL of your cultures into **labelled** microcentrifuge tubes. Collect the cells by centrifugation for 1 minute using the microcentrifuge at top speed. Remove and discard the supernatant.

4. Rinse the cells. Add 1 mL deionized water to each tube. Resuspend the cell pellets by gently drawing the cells in and out of a micropipette tip, taking care to prevent premature lysis of the cells. This rinse step removes proteins from the culture medium that may contaminate the cell pellet. Centrifuge again for 1 minute at top speed.

5. Discard the supernatant and resuspend the cells in 100 µL deionized water.
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**Prepare the protein extract**

6. Add 100 µL of 0.2N NaOH to each tube, and incubate the cells for 5 minutes at room temperature. (The addition of NaOH does not lyse the cells, but it makes them more permeable and more fragile.)

7. Pellet the cells again in the microcentrifuge. **Remove and discard the supernatant. (The proteins that you are interested in are in the pellet.)**

8. **Resuspend the cells** in 50 µl 2 X SDS-PAGE sample buffer.* **Ensure that the tubes are tightly capped.** IMMEDIATELY place the tubes in a boiling water bath. Leave the cells in the water bath for 3 minutes. This treatment effectively denatures the proteins. Yeast cells contain many proteases that could degrade other cellular proteins, so it's important that the proteases are denatured before they have the chance to attack other cellular proteins.

    **NOTE:** The 2 X SDS-PAGE sample buffer contains 2-mercaptoethanol (also known as β-mercaptoethanol, or BME). Use appropriate caution and work quickly when handling this reagent. **BME is a volatile reagent with a strong odor reminiscent of rotten fish.**

9. After boiling, store the samples in the freezer for future use.

*2 X SDS-PAGE sample buffer consists of:
- 120 mM Tris/HCl, pH 6.8
- 10% glycerol (glycerol is significantly more dense than water)
- 4% SDS
- 8% 2-mercaptoethanol
- 0.004% bromophenol blue (a tracking dye for electrophoresis)

**References**


