Chapter 3

Isolation of Mitochondria from *Saccharomyces cerevisiae*

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Abstract

The budding yeast *Saccharomyces cerevisiae* is an important model organism to study cellular structure and function. Due to its excellent accessibility to genetics and biochemical and microscopic analyses, studies with yeast have provided fundamental insights into mitochondrial biology. Yeast offers additional advantages because it can grow under fermenting conditions when oxidative phosphorylation is not obligatory and because the majority of mitochondrial structure and function are largely conserved during evolution. Isolation of mitochondria is an important technique for mitochondrial studies. This chapter focuses on procedures for the isolation and purification of intact yeast mitochondria that can be used for numerous functional assays as well as for analyses of mitochondrial ultrastructure.

Key words Yeast culture media, Spheroplasts, Homogenization, Cellular fractionation, Isolation and purification of mitochondria

1 Introduction

Mitochondria are essential organelles of eukaryotic cells. They exert a multitude of important metabolic functions including energy transduction by the citrate cycle and oxidative phosphorylation, metabolism of amino acids and fatty acids, and synthesis of FeS clusters, heme, quinones, and phospholipids. They are involved in apoptosis and developmental processes such as aging and play an important role in many important human disorders, including diabetes, cancer, and neurodegenerative disorders, such as the Alzheimer, Parkinson, and Huntington diseases.

The budding yeast *Saccharomyces cerevisiae* is a powerful model organism to study mitochondrial function and architecture. Since protein composition, function, and architecture of mitochondria are highly conserved among eukaryotes, studies of yeast mitochondria are instructive for understanding the molecular biology of mitochondria also of mammalian cells. In addition, since *Saccharomyces cerevisiae* can satisfy its energy requirement by fermentation, a number of viable knockout mutants for the genes required for respiration can be constructed. This makes it much

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easier to analyze the function of mitochondrial proteins essential for respiration. Furthermore, *Saccharomyces cerevisiae* offers excellent accessibility to genetic manipulation and biochemical and macroscopic analyses. Indeed, a large number of conserved proteins and complexes required for mitochondrial function and architecture were first discovered using yeast cells.

Saccharomyces cerevisiae can utilize both fermentable and nonfermentable carbon sources. Depending on carbon source availability, the cells regulate the quantity and architecture of mitochondria [1–3]. Glucose is a routinely used fermentable carbon source. On glucose-containing medium, most of ATP is produced in the cytosol by fermentation. Under these conditions the expression of a number of mitochondrial proteins including enzymes of the citrate cycle and oxidative phosphorylation are strongly repressed, as well as is the formation of a highly branched mitochondrial network. Galactose is another fermentable carbon source; however, it does not repress induction of mitochondrial proteins. In contrast, on nonfermentable carbon source such as glycerol or lactate, the cells produce ATP for growth exclusively in the mitochondria by oxidative phosphorylation. Under these conditions, expression of mitochondrial proteins is highly induced, and mitochondria form an extensive, highly branched network.

Isolation of mitochondria is a fundamental and important technique for mitochondrial research. Isolated mitochondria can be used for many purposes, e.g., isolation and characterization of mitochondrial proteins, analysis of the topology and modification of mitochondrial proteins, and in vitro protein import [4–7]. Critical steps during the isolation process are the homogenization of cells and their separation from other organelles by cell fractionation [8]. The following quality criteria must be taken into consideration: yield, purity, and intactness of mitochondria. Many downstream procedures require substantial amounts of mitochondria. In our laboratory, mitochondria are typically isolated from the cells grown in 500–2000 ml of liquid medium when OD_{600} is around 1. The choice of carbon source is also important. Higher yields of mitochondria can be obtained when the cells are grown on nonfermentable carbon sources such as glycerol or lactate. The purity of the isolated mitochondria is particularly important when clear separation of mitochondria from other organellar membranes is required. This further step is usually performed by density gradient centrifugation [9]. Several measures to maintain the functional intactness of yeast mitochondria are described.

2 Materials

2.1 Growth Media

1. YP medium: Dissolve 10 g Bacto yeast extract and 20 g Bacto Peptone in 800 ml of water. Adjust pH to 5.5 with potassium hydroxide and fill up to 900 ml. Sterilize by autoclaving.

2. Synthetic minimum (S) medium: Dissolve 6.7 g of yeast nitrogen base without amino acids in 800 ml water. Adjust pH to 5.5 with potassium hydroxide and fill up to 900 ml. Sterilize by autoclaving.
3. Dropout mix: Mix the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, methionine, isoleucine, phenylalanine, proline, serine, threonine, tyrosine, valine, myoinositol (2 g each), and p-aminobenzoic acid (0.2 g). For storage, homogenize the mixture using a mortar and pestle.
4. Synthetic complete (SC) medium: Dissolve 6.7 g of yeast nitrogen base without amino acids and 1.3 g of dropout mix in 800 ml water. Adjust pH to 5.5 with potassium hydroxide and fill up to 900 ml. Sterilize by autoclaving.
5. 50× auxotrophic markers: Dissolve 112 mg of uracil, 1095 mg of leucine, 410 mg of tryptophan, 314 mg of histidine, 203 mg of adenine, and 900 mg of lysine in 90 ml of water, and fill up to 100 ml (*see* **Note 1**). Sterilize by filtration. Omit the auxotrophic marker(s) used for selection of transformants.
6. 10× carbon sources: Glucose, galactose, glycerol, or lactate can be used as a carbon source. To make 20% (w/v) glucose, dissolve 20 g of glucose in 80 ml of water and fill up to 100 ml. To make 30% (w/v) galactose, dissolve 30 g of galactose in 70 ml of water and fill up to 100 ml. To make 30% (v/v) glycerol, fill up 30 ml of glycerol to 100 ml with water. To make 20% (v/v) lactate, dilute 20 ml of lactic acid with 30 ml of water, adjust pH to 5.5 with potassium hydroxide, and fill up to 100 ml. Sterilize all solutions by autoclaving.
7. Growth media: Mix 900 ml of YP, S, or SC medium and 100 ml of 10× carbon source. For S and SC media, add 20 ml of 50× auxotrophic markers.

2.2 Isolation of Mitochondria

1. Alkaline solution: 100 mM Tris–SO_4 pH 9.4, 10 mM DTT (*see* **Note 2**).
2. Spheroplast buffer: 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1.2 M sorbitol.
3. 20 × zymolyase buffer: 20 mg/ml of zymolyase 20 T, 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1.2 M sorbitol (*see* **Note 3**).
4. Lysis buffer: 20 mM MOPS-KOH pH 7.2, 1 mM EDTA, 0.6 M sorbitol, 0.2% (w/v) BSA, 1 mM PMSF (*see* **Note 4**).
5. SEM buffer: 20 mM MOPS-KOH pH 7.2, 1 mM EDTA, 0.6 M sorbitol.

2.3 Purification of Mitochondria by Sucrose Gradient Centrifugation

1. SEM buffer: 20 mM MOPS-KOH pH 7.2, 1 mM EDTA, 0.6 M sorbitol.
2. 60% sucrose buffer: 60% sucrose (w/v), 20 mM MOPS-KOH pH 7.2, 1 mM EDTA.

3. 32% sucrose buffer: 32% sucrose (w/v), 20 mM MOPS-KOH pH 7.2, 1 mM EDTA.
4. 23% sucrose buffer: 23% sucrose (w/v), 20 mM MOPS-KOH pH 7.2, 1 mM EDTA.
5. 15% sucrose buffer: 15% sucrose (w/v), 20 mM MOPS-KOH pH 7.2, 1 mM EDTA.

3 Methods

3.1 Growth of Yeast Cells

YP is a rich medium, routinely used for yeast growth. S and SC media are used for selection of yeast transformants for auxotrophic markers. S medium is the synthetic minimum medium composed of salts, trace elements, vitamins, and nitrogen source. Addition of dropout mix (mixture of amino acids) to S medium can improve yeast growth [10]. For selection of yeast transformants, uracil, leucine, tryptophan, histidine, adenine, and lysine are typically used as auxotrophic markers. In this protocol, these amino acids are omitted from dropout mix. Depending on auxotrophic marker used for transformant selection, appropriate amino acids should be added.

Budding yeast can utilize a variety of carbon sources. Glucose and galactose are fermentable carbon sources, and glycerol and lactate are nonfermentable carbon sources. Glucose, but not other carbon sources, represses the expression of a number of mitochondrial proteins as well as the formation of a highly branched mitochondrial network [1–3]. Selection of the suitable carbon source depends on the scientific question asked. In this section, the regular procedure for cultivation of wild-type yeast cells in 500 ml of YP glycerol medium is described.

1. Inoculate yeast colony with the size of 2–3 mm in diameter from an agarose plate medium in 20 ml of YP glycerol medium and cultivate for 2–3 days at 30 °C with shaking. The cells are in the stationary phase when OD_{600} reaches around 6–7 (*see* **Notes 5** and **6**).
2. Dilute yeast pre-culture in 500 ml of YP glycerol medium and cultivate cells at 30 °C with shaking until OD_{600} reaches 1 (*see* **Notes 7** and **8**).
3. Harvest cells by centrifugation at 2000 × *g* for 5 min at room temperature for isolation of mitochondria (*see* **Note 9**).

3.2 Isolation of Crude Mitochondria

The procedure consists of three steps: (1) preparation of spheroplasts, (2) homogenization of spheroplasts, and (3) isolation of mitochondria by differential centrifugation. Two different procedures for homogenization of spheroplasts are described. In the classical method, the spheroplasts are homogenized using a Dounce homogenizer [8]. In the alternative method, instead, the spheroplasts are

homogenized by repeated pipetting without using homogenizer [11]. This alternative method may reduce the yield of mitochondria but significantly improves intactness of mitochondria, which is crucial for analysis of mitochondrial ultrastructure.

3.2.1 Preparation of Spheroplasts

Yeast cells have cell walls mainly composed of β-1,3-d-glucan, β-1,6-d-glucan, chitin, and mannoproteins [12]. For efficient homogenization, yeast cells need to be converted into spheroplasts by digestion of cell walls. To this end, zymolyase is typically used. Zymolyase is produced from *Arthrobacter luteus* and contains an essential enzyme, β-1,3-glucan laminaripentaohydrolase, for lysis of yeast cell walls [13, 14]. Because cell wall lysis can lead to the break of plasma membranes that is triggered by the high internal turgor pressure, the buffer contains sorbitol to protect cells from osmotic stress.

1. Resuspend the harvested cells with 20 ml water.
2. Harvest the cells by centrifugation at 2000 × *g* for 5 min at room temperature.
3. Measure the wet weight (g) of the cells (*see* **Note 10**).
4. Resuspend the cells in 10 ml alkaline solution (*see* **Note 11**).
5. Incubate the cells at 30 °C for 10 min with gentle shaking.
6. Harvest the cells by centrifugation at 2000 × *g* for 5 min at room temperature.
7. Resuspend the cells with 10 ml spheroplast buffer per gram wet weight of cells.
8. Add 500 µl 20× zymolyase buffer per gram wet weight of cells.
9. Incubate cells at 30 °C for 20–40 min with gentle shaking (*see* **Note 12**).
10. Harvest the spheroplasts by centrifugation at 2000 × *g* for 5 min at 4 °C. All further steps should be done at 4 °C.
11. Wash the spheroplasts briefly with 30 ml ice-cold lysis buffer. It is not necessary to pipet to homogeneity. Just invert the centrifuge bottle several times.
12. Harvest the spheroplasts by centrifugation at 2000 × *g* for 5 min.
13. Repeat **steps 11** and **12**.
14. Go to Subheading 3.2.2. for the classical homogenization method using Dounce homogenizer or go to Subheading 3.2.3**.** for the gentle homogenization method by pipetting.

3.2.2 Homogenization of the Spheroplasts with Dounce Homogenizer

All steps should be performed at 4 °C using precooled buffers and glassware and plastic ware. It is also important to precool all rotors and centrifuges used.

1. Resuspend the pellet of spheroplasts in 20 ml ice-cold lysis buffer and transfer it to a 50-ml Dounce homogenizer.
2. Homogenize the spheroplasts with 15 strokes.
3. Centrifuge the homogenate at 2000 × *g* for 5 min and transfer the supernatant to a new tube.
4. Repeat **steps 1–3** with the pellet from the **step 3**.
5. Combine the supernatants to a tube and centrifuge at 12,000 × *g* for 10 min. Remove the supernatant.
6. Resuspend the pellet containing mitochondria in 1 ml ice-cold SEM buffer by gentle pipetting using a P1000 tip with 1 cm cutoff, and fill up to 30 ml with ice-cold SEM buffer.
7. Centrifuge again at 12,000 × *g* for 10 min. Remove the supernatant.
8. Resuspend the pellet in 500 μl ice-cold SEM buffer by gentle pipetting using a P1000 tip with 1 cm cutoff.
9. Take an aliquot to determine protein concentration.
10. Make single-use aliquots (typically 30–50 μl) and freeze them in liquid nitrogen. Store at −80 °C (*see* **Note 14**).

3.2.3 Homogenization of the Spheroplasts by Pipetting

All steps should be performed at 4 °C using precooled buffers and glassware and plastic ware. It is also important to precool all rotors and centrifuges used.

1. Add 10 ml ice-cold lysis buffer to the harvested spheroplasts.
2. Resuspend the spheroplasts by repeated pipetting using a P5000 tip with 1 cm cutoff. Pipet gently 40 times.
3. Centrifuge at 2000 × *g* for 5 min and collect the supernatant to a new tube.
4. Add 10 ml ice-cold lysis buffer to the pellet.
5. Repeat **steps 2** and **3**.
6. Combine the supernatants to a tube, and centrifuge at 2000 × *g* for 5 min to remove remaining cell debris. Collect the supernatant to a new tube.
7. Centrifuge the supernatant at 12,000 × *g* for 5 min. Remove the supernatant.
8. Wash the pellet containing mitochondria in 20 ml ice-cold SEM buffer by gentle pipetting using a P5000 tip with 1 cm cutoff.
9. Centrifuge at 12,000 × *g* for 5 min. Remove the supernatant.
10. Resuspend the mitochondrial pellet in 500 μl ice-cold SEM buffer by gentle pipetting using a P1000 tip with 1 cm cutoff.
11. Take an aliquot to determine protein concentration (*see* **Note 13**).

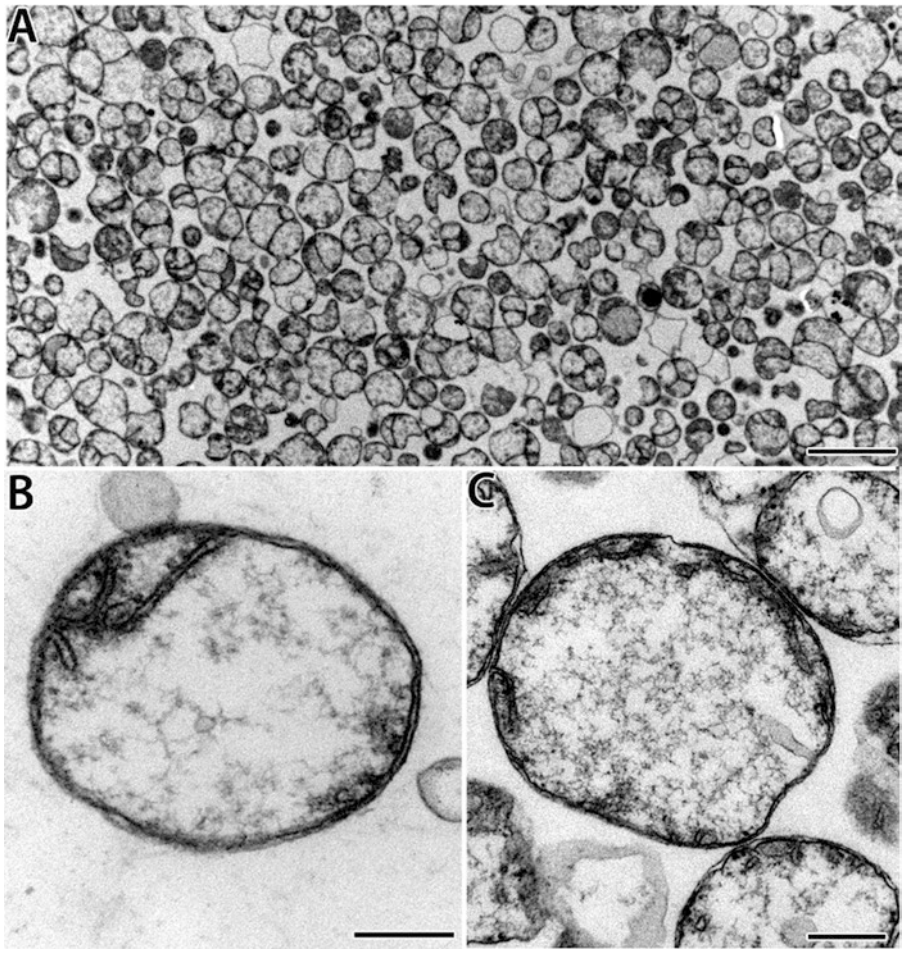

Fig. 1 Electron microscopic analysis of isolated mitochondria of *S. cerevisiae* (YPH499). (**a**) Overview (magnification 7000-fold). (**b** and **c**) Ultrastructure of single mitochondria. Scale bars: 0.2 μm

12. Make single-use aliquots (typically 30–50 μl) and freeze them in liquid nitrogen. Store at −80 °C (*see* **Note 14**).

Electron microscopy images of the mitochondria isolated by homogenization of spheroplasts by pipetting are shown in Fig. 1.

3.3 Purification of Mitochondria by Sucrose Gradient Centrifugation

All steps should be performed at 4 °C using precooled buffers and glassware and plastic ware. It is also important to precool all rotors and centrifuges used.

Isolation of crude mitochondria as described above can provide sufficient quality of mitochondria, e.g., for the topological analysis of mitochondrial proteins and in vitro protein import analysis. However, the crude mitochondria contain significant amounts of non-mitochondrial membranes such as ER and vacuolar membranes. Several applications may need the separation of mitochondria from ER and vacuolar membranes. In the following, a method,

based on a previously published procedure [9], for the purification of mitochondria using sucrose density gradient is described.

1. Homogenize the crude mitochondrial fraction (5 mg/ml in SEM) by 10 strokes in a glass-Teflon potter.
2. Prepare a four-step sucrose gradient in a SW41 rotor tube. For this, pipet 1.5 ml 60% sucrose buffer to a tube, carefully overlay it with 4 ml 32%, then 1.5 ml 23%, and finally 1.5 ml 15% sucrose buffer.
3. Load the crude mitochondrial fraction onto the sucrose gradient.
4. Centrifuge at 134,000 × *g* in a Beckman SW41 Ti rotor for 1 h at 4 °C.
5. Collect the purified mitochondrial fraction from the 60/32% interface.
6. Dilute the recovered mitochondrial fraction with 2 volumes of SEM and centrifuge at 10,000 × *g* for 15 min at 4 °C.
7. Resuspend the pellet in ice-cold SEM buffer by gentle pipetting using a P1000 tip with 1 cm cutoff.
8. Take an aliquot to determine protein concentration.
9. Make aliquots of 30–50 μl and freeze them in liquid nitrogen. Store at −80 °C.

4 Notes

1. The amino acids can be dissolved in water at 50 °C.
2. DTT should be added in the buffer just before use. 1 M DTT stock solution can be stored at −20 °C.
3. Zymolyase 20 T should be dissolved in the buffer just before use.
4. PMSF and BSA should be added in the buffer just before use. Prepare a stock of 100 mM PMSF in isopropanol and store it at −20 °C. PMSF is not soluble in water at high concentration.
5. Wild-type cells can be stored on YP glucose, galactose, or glycerol agarose plate at least for 1 month at 4 °C.
6. High mitochondria yields can be obtained by cultivating yeast cells in YP medium with glycerol, lactate, or galactose. Cultivation of yeast cells in YP medium with glucose represses mitochondrial gene expression and therefore reduces mitochondrial yield. If the mutant strain is defective in respiratory function or lacking mitochondrial DNA but is viable in fermentable carbon sources, galactose or glucose medium can be used as a carbon source. If the mutant strain shows temperature-sensitive phenotype, the cells can be cultivated at 23 °C instead of 30 °C.

7. The growth rate of the cells is different dependent on the yeast strain used. To estimate when OD_{600} will reach 1, a preliminary experiment for the growth rate is recommended.
8. In our laboratory, the pre-culture in the stationary phase is diluted with fresh medium to OD_{600} less than 0.2 to allow cells to grow more than two generations.
9. For isolation of mitochondria, freshly harvested cells should be used.
10. Approximately 0.5 g of the cells is obtained from 500 ml culture. The easiest way to determine the wet weight of cells is to weigh an empty centrifuge tube, use it to harvest the cells, and then determine the weight of the same tube with cell pellet after centrifugation. The wet weight of the cells represents the difference between two measurements.
11. The cells are resuspended in 10 ml of alkaline solution irrespective of the wet weight of the cells.
12. To control spheroplast formation, add 10 µl of the resuspended cells in 1 ml of water before and after zymolyase treatment. When the cells are converted into spheroplasts sufficiently, OD_{600} is decreased to approximately 5–10% of that before the addition of zymolyase. Do not increase the incubation time with zymolyase to longer than 1 h.
13. Usually the concentration of crude mitochondria is 5–10 µg/µl. The concentration of mitochondria can be adjusted by adding SEM buffer.
14. Frozen mitochondria can be used for many applications such as in vitro protein import assay. However, some applications require freshly isolated mitochondria. Sub-fractionation of the mitochondria is one such example [4].

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