CHAPTER 5

Protein Extraction from Fungi

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1. Introduction

In order to study proteins from yeasts and filamentous fungi, it is important to consider a number of basic features of the organisms. First, filamentous fungi undergo a growth cycle that includes differentiation and compartmentalization. In addition, both the filamentous fungi and yeasts will age during growth, and older cultures will undergo autolysis. As a result, particular proteins may only be associated with one part of the growth cycle, such as sporulation or autolysis, and this must be taken into account in determining growth conditions and sampling times.

Second, many of the enzymes produced during the growth period are sequential and may either be subject to significant repression or require induction by a substrate or substrate component. Examples of this include the requirement for chitin or chitin-like components to induce chitinases *(1)*, and the repression of some fungal proteases by glucose *(2)*.

Third, fungi possess rigid cell walls and complex cell-wall/membrane systems *(3)*. It is therefore important to ascertain the potential location of proteins prior to their extraction, since cell-wall-associated and extracellular proteins will be lost during intracellular extractions. An example of this is the utilization of many of the traditional fungal nutrients, such as cellulose and lignin, where significant levels of extracellular enzymes will be produced. Most of these extracellular enzymes can be produced in sufficient concentrations for them to be purified and characterized directly from the spent growth medium *(4,5)*.

A simple growth and extraction procedure is described here. This is a standard regime that will allow the extraction of intracellular proteins

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from a wide range of filamentous fungi, and has been used successfully with many fungal genera, including *Fusarium, Ganoderma, Aspergillus, Colletotrichum, Beauveria, Phoma, Verticillium,* and *Metarhizium (6).* The method has not been optimized toward any particular fungal group, and has proven suitable for filamentous ascomycetes and basidiomycetes as well as yeasts *(7–9).* The major variation that will be needed for different fungal groups is the growth medium and the length of the growth period (*see* Notes 1 and 2). Although a crude method, extracts produced in this way retain sufficient integrity and activity for enzyme assays and isoenzyme electrophoresis. An additional feature of this method is that the spent culture fluid may be retained for the detection of extracellular enzymes. Initially, this will only contain a small number of glucose-independent enzymes, but as the culture grows and the free glucose concentration decreases, further enzymes can be detected or extracted *(7,10)*

2. Materials

Fungal growth media and buffers should be sterilized prior to use. Growth media and buffers can routinely be sterilized at 10 psi for 10 min in a benchtop autoclave. Although the materials listed here are unaffected, is should be remembered that in complex media and buffers, individual components may break down or react during autoclaving, and so may need to be individually filter sterilized.

1 Malt extract agar (MEA). 20 g Malt extract (Oxoid, Basingstoke, UK), 1 g peptone (Oxoid; Bacteriological), 20 g glucose, 15 g agar, 1 L distilled water *(11)*
2 Glucose yeast medium (GYM). 1g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g glucose, 1 mL 0.5% aqueous $CuSO_4$ $5H_2O$, 1 mL 1% aqueous $ZnSO_4$ $7H_2O$, distilled water to 1 L *(7)*
3. Pectin broth: 0.9 g $NH_4H_2PO_4$, 2g $(NH_4)_2HPO_4$, 0.1 g $MgSO_4$ $7H_2O$, 0.5 g KCl, 10 g citrus pectin (Sigma, Poole, UK), 1 L distilled water.
4 Tris-glycine buffer: 3 g Trizma (Sigma, Poole, UK), 14 4 g glycine, 1 L deionized water, pH 8.3.
5. Pectinase gel: 0.2 g Citrus pectin, 10 g acrylamide, 0.25 g methylene-bisacrylamide, 0.1 mL TEMED (*N,N,N',N'*-tetramethylethylenediamine), 0.1 g ammonium persulfate, 100 mL gel buffer.
6 Gel buffer: 0.525 g Citric acid monohydrate, 4.598 g Tris, 1 L deionized water.
7. Electrode buffer: 7.22 g Boric acid, 15.75 g sodium tetraborate decahydrate, 1 L deionized water
8 0.1*M* Malic acid.

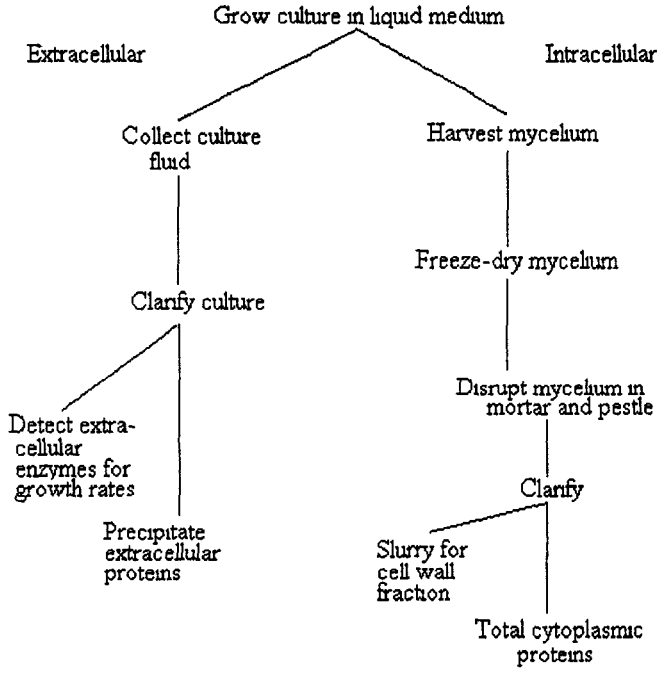

Fig. 1. Schematic diagram of the complete protocol.

9 0.015% Aqueous ruthenium red.
10. Sterile deionized water.

3. Methods

The method presented here will enable the extraction of intracellular proteins and electrophoresis of extracellular enzymes from filamentous fungi and yeasts (Fig. 1). The Notes section details further considerations that may be needed for specific organisms or extractions

3.1. Extraction of Intracellular Proteins from Metarhizium

The following protocol describes the extraction of aqueous intracellular proteins from the filamentous fungus *Metarhizium anisopliae*. Further details regarding growth and extraction conditions for other filamentous fungi are given in the Notes 2–7.

1. Grow *Metarhizium* culture on malt extract agar for 7 d at 25–28°C.
2. Remove a plug (approx 0 5-cm diameter) of culture from the agar plate with a flamed cork borer or scalpel. Cut the plug into at least 10 smaller

pieces with a flamed scalpel and inoculate into 10-mL sterile GYM in a 28-mL Universal bottle or small flask (*see* Note 3).

3. The 10-mL culture is a starter culture for the subsequent main growth period and is required to ensure an actively growing inoculum Incubate the starter culture at 25–28°C in an incubator, with shaking, for 60–72 h.
4. Aseptically transfer the starter culture into a 250-mL conical flask containing 60 mL of fresh sterile GYM. Replace the flask in the incubator and continue incubation with shaking for a further 60–72 h (*see* Note 4).
5. Harvest the mycelium from the flask by vacuum-assisted filtration. A Buchner funnel containing Whatman No. 3 filter paper is suitable for this purpose (*see* Note 5). Take care to ensure that any aerosols that may be formed from the filtration or the vacuum pump are minimized and contained.
6. Wash the mycelium once in the Buchner funnel with sterile deionized water, and transfer the harvested mycelium from the filter paper to a plastic Petri dish with a flamed or alcohol-sterilized spatula.
7 Freeze the mycelium at –20°C for storage prior to extraction.
8. Prepare the mycelium for extraction by freeze-drying for 24 h. This is one of several possible methods of obtaining cell breakage (*see* Notes 6 and 7).
9. Disrupt the freeze-dried mycelium by briefly grinding it in a mortar and pestle (*see* Note 6) The ground mycelium should be collected in sterile 1.5-mL microcentrifuge tubes in approx 500-mg amounts. This is roughly equivalent to the conical portion of the tube. These tubes of ground mycelium can be stored at –20°C for at least 3 mo.
10. Rehydrate 500 mg of ground mycelium in 1 mL of Tris-glycine buffer (*see* Note 8). This will need mixing with a micropipet tip or Pasteur pipet to form an even slurry.
11. Clarify the slurry by centrifugation at 12,500*g* for 40 min at 4°C After centrifugation, collect the supernatant into another sterile microcentrifuge tube (*see* Note 9).
12. The collected supernatant will contain the total cytoplasmic proteins. The samples as prepared here typically contain 15–100 mg/mL protein (*see* Note 10). The extracts can be used directly in gel electrophoresis or column chromatography. Alternatively, standard precipitation techniques can be used to purify specific proteins further (*see* Note 9).

3.2. Electrophoresis of Extracellular Enzymes

The following method describes the induction and electrophoresis of extracellular pectinases. Pectin is used, both in the production medium and in the polyacrylamide gel, to induce the enzymes and to avoid the necessity for subsequent overlays for staining. The method given here was originally described by Cruickshank and Wade *(4),* and is given here as in

Paterson and Bridge *(6)*. The method has successfully been used without modifications with *Fusarium, Penicillium, Colletotrichum, Ganoderma,* and *Rhizoctonia.* A horizontal gel system is required, and self-adhesive vinyl tape (Dymo) is used to produce wells in a simple gel mold. Sephadex is used in the sample wells to make more well-defined enzyme patterns (*see* Note 11). Bromophenol blue is not used to measure the buffer front, since it can be seen adequately without the dye. This type of pectin/acrylamide gel gives enzyme patterns that are asymmetrical, unlike conventional isozyme bands. This is probably because of more definite chromatographic adsorption effects from charged pectin molecules, which are dissolved in the gel, i.e., the protein molecules are interacting with the gel.

1. Inoculate cultures into 2 mL pectin broth (PB) in a 5 mL Bijoux bottle.
2. When the cultures have grown sufficiently (usually 7 d), remove 200 µL of the culture fluid, and suspend in superfine (G-25) Sephadex at a concentration of 20 mg/200 µL.
3. Prepare a pectin-acrylamide gel for horizontal gel electrophoresis (*see* Note 11). Use three pieces of Dymo tape (2.5 × 5.0 mm) to form a well with a volume of 20 µL in the center of a gel mold. Similar pieces of tape are placed 1.25 cm apart across the mold to form the other wells.
4. Set up the horizontal electrophoresis with anode and cathode buffer compartments containing gel buffer. Use filter paper wicks to form the "contact" between the buffer and the gel
5 Place the negative electrode 3.5 cm below the sample wells to allow adequate migration of any positively charged pectinase component
6. After electrophoresis, submerge gels in 0.1*M* malic acid for 1 h, rinse in distilled water, and stain overnight in 0.015% (w/v) ruthenium red at 4°C. Rinse the gel for 1 h with three changes of distilled water.
7. Enzyme patterns are recorded by:
   a. Tracing the gel and enzyme patterns onto vinyl transparencies and photocopying the tracing;
   b. Photographic recording; and/or
   c. Direct comparison from dried gels.
8. Polygalacturonase (PG) activity gives transparent zones, pectin esterase (PE) activity gives dark zones, and pectin lyase (PL) gives yellow zones.

4. Notes

1. The growth medium selected will vary depending on the requirements of the fungus under study. The GYM medium given in the Section 2. is a general-purpose medium suitable for the growth of many filamentous ascomycetes and basidiomycetes. For organisms that may be more fastidious, a

richer medium, such as peptone yeast extract glucose *(12)*, can be very useful (*see* ref *6*). Oomycetes, such as *Pythium* and *Phytophora*, may require either a source of sterol or a more complex organic medium, such as V8 medium *(11)*. Organic components in growth media can vary between suppliers, and this variation may affect the biochemical properties of the fungus *(13)*. It is therefore important to standardize organic components, such as yeast extract, by only using a single source of supply.

2. Growth conditions are important in obtaining a reliable and constant source of protein, and it is usually desirable to extract from cultures consisting of material of a constant age. This is obtained by first inoculating fungal cultures into small starter cultures of the main growth medium. These starter cultures are incubated, usually as shaken liquids, and provide the inoculum for the main growth phase. Although the exact conditions will vary depending on the organism studied, a typical starter culture would consist of 10 mL of GYM medium in a 25–30 mL vessel, incubated on an orbital shaker at 25°C for 60 h. The entire starter culture is then used as inoculum for 60 mL of GYM medium in 250-mL conical flasks, which is then incubated at 25°C on the shaker for 3–5 d. Growth periods and temperatures will depend on the rate of growth and differentiation of the fungus studied Most yeast cultures will produce sufficient suitable biomass after incubation at 30°C under a growth regime of 24 h in a starter culture and 24 h in a shaken flask. Many filamentous fungi however require 60 h in a starter culture followed by 3–5 d in shaken flasks at 25°C. In order to maintain reproducibility, incubation temperatures should be kept constant.
3. The inoculum used for the initial starter cultures is important and should contain a large number of potential "growing" points. That is, the inoculum should ideally be particulate so that mycelial or yeast growth can occur at many different points. This presents no problems if the culture is naturally particulate, such as yeast cells, or if conidial suspensions can be used (e.g., ref. *5*). However, if the fungus grows only as a mycelial mat, an inoculum made from broken mycelia will give a more homogenous and greater biomass than one derived from plugs or "lumps" of culture.
4. In many cases, the proteins of interest will be associated with active growth of the fungus. It is therefore often necessary to establish some form of growth curve for the organism prior to harvesting the growth. The growth rate of yeasts, and fungi growing in a yeast-like phase, can be estimated by sampling and measuring the turbidity of the growth medium over a period of time *(14)*. However, with filamentous organisms, this becomes impractical. One alternative is to assay the culture medium for the extracellular enzymes β-glucosidase, β-galactosidase, and diacetyl-chitobiosidase. In many fungi, these enzymes are each associated with particular features of the growth

cycle when grown in glucose-containing media. β-glucosidase is generally produced in the early phases of growth, β-galactosidase is not produced until the glucose concentration has been reduced, and diacetyl-chitobiosidase is generally produced during autolysis. These activities can be readily screened directly from culture fluids with the substrates 4-methylumbeliferyl β-D-glucoside, 4MU β-D-galactoside, and 4MU β-D-*N,N'*-diacetyl-chitobioside *(6,10)* The substrates can be kept as 50-m*M* solutions in dimethylformamide, and for use, the stock solutions are diluted 0.15:9.85 mL in 0.05*M* sodium acetate, pH 5.4. Activity is determined by adding 50 µL of culture fluid to 50 µL of each substrate and incubating these for 4 h at 37°C. Positive enzyme activity can then be detected as fluoresence under UV light after the addition of 50 mL of saturated aqueous sodium bicarbonate.

5. When the required phase of growth has been reached, the fungal biomass must be harvested from the growth medium. Again methods differ depending on the organism, but in general, yeasts can be harvested by centrifugation at 3–7000*g*, whereas filamentous fungi are better harvested by vacuum filtration onto Whatman No. 3 filter papers in a Buchner funnel. In both cases, the resulting biomass should be washed in sterile distilled water to remove any residues from the media. If extracellular proteins, such as extracellular enzymes, are required, these can be extracted directly from the culture fluid after the mycelium has been harvested. Typically, the culture fluid is clarified by centrifugation and proteins extracted by one of the general protein extraction techniques, such as precipitation with acetone or methanol/ammonium or by immunoprecipitation *(15,16)*.
6. Efficient cell breakage is necessary to ensure a good recovery of proteins from filamentous fungi. A wide variety of cell breakage methods have been reported in the literature, varying from enzymatic digestion of the cell wall to physical disruption procedures, such as grinding mycelium in a mortar and pestle *(6)* or homogenizing thick cell pastes *(17)*. Enzymatic digestion has been used to generate protoplasts, which can be harvested from the remaining cell debris. The protoplast preparations can then be lysed to release intracellular proteins *(18,19)* Many fungi produce active intra- and extracellular proteinases, and these are usually active under the conditions used for enzymatic digestion of cell walls. As a result, physical disruption of an "inert" sample is generally preferred. Protease activity has been supressed by phenylmethylsulfonylfluoride (PMSF) in some cases *(20)*. Fungal cell walls can be disrupted by briefly grinding freeze-dried mycelium in a mortar and pestle. Additional abrasives are not usually required, although carborundum may be added if required. This procedure should not be performed on an open bench owing to the possible hazardous nature of the dust produced. A single flask of 60 mL actively growing culture will

give about 500–2000 mg of freeze-dried material. The method of grinding a freeze-dried culture as described here is quick and reliable, but requires access to freeze-drying equipment. A commonly used alternative is to grind the harvested mycelium in liquid nitrogen *(15)*. However, appropriate safety measures should be considered to avoid any potential contact or splashing from the liquid nitrogen

7. Yeast cells can be disrupted by passage through a pressure cell, such as a French Press *(21)*, although three or more passages may be necessary to achieve 70% breakage. Yeast cells can also be broken by shaking frozen cultures with glass beads. The glass beads used are generally larger than the Ballotini beads used for bacteria and 2–2.5-mm beads can produce adequate disruption after 10–15 min of shaking. This method can generate sufficient local heating to denature some proteins, so effective cooling of the system is required
8. The ground mycelium is rehydrated in the method at 500 mg in 1 mL of Tris-glycine buffer This will need mixing to form an even slurry. In some cases, detergent-containing buffers have been shown to give higher yields of total protein *(22,23)*, but the simple Tris-glycine buffer used here is adequate for general-purposes.
9. The total aqueous extract will contain the cytoplasmic proteins. Normally, the pellet of unbroken cells and debris is discarded although this can be saved if the cell-wall fraction is required. This extract will, however, contain many other components, including polysaccharides. The degree of further purification necessary will depend on the protein and level of activity required, as well as the level of polysaccharide contamination. As mentioned, the supernatant contains the total cytoplasmic proteins and may be used directly. Proteins in these crude extracts can be readily separated by polyacrylamide gel electrophoresis *(7,8)*, and this technique can be used to provide cell-free total protein patterns or, with specific stains, to demonstrate particular enzymes. Alternatively, it may be necessary to clarify the supernatant further by a second centrifugation or filtration through a 0.45-μm filter. Specific proteins can then be purified through standard precipitation techniques as mentioned in Note 5. Further clarification and separation, generally by differential centrifugation in sucrose density gradients will be required to extract the cell-wall fraction *(23)*
10. The protein content of the supernatant should be estimated by one of the standard protein determination methods, such as Lowry's determination *(24)*
11. Under the conditions used, pectinase isoenzymes may be both positively and negatively charged. It is therefore very important to undertake the electrophoresis in a horizontal gel system that allows for the construction of central sample wells

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