

An improved method for protein extraction from minuscule quantities of fungal biomass

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Abstract

Filamentous fungi are ubiquitous eukaryotes having chitin as a major constituent of the cell wall. Chitin is tough to lyse due to which the intracellular fungal proteins are not readily accessible. The problem is further enhanced when the biomass to be analyzed for protein studies is too little due to the extreme experimental parameters under consideration such as increased or lowered pH, temperature, hydrostatic pressure, nutrients, etc. The method described here is capable of obtaining proteins from minuscule quantities of biomass (~5 mg lyophilized biomass). In this study, different lysing conditions and varied composition of extraction buffers were tried to obtain maximum protein of high quality. Lysis with zirconium beads in a combination buffer system (Tris-MgCl₂ buffer, Urea buffer I and Urea buffer II) was best for extracting proteins from the fungal isolates used. The protocol described here provides for a simple and quick method for extraction of high-quality proteins from very less biomass that could be extended to other tough to lyse biological material also.

Keywords: Fungi, Homogenizer, Biomass, Protein, Zirconium beads

Protein extraction is the most critical step of proteomic studies, which often differs with the type of organism and requires thorough standardization to obtain good quality proteins for analysis. Extraction of proteins from filamentous fungi has always posed challenges due to the robust, chitinous cell wall. Although liquid nitrogen crushing of fungal mycelia is the most commonly used method for extracting proteins [1, 2, 3], it has limitations when the amount of biomass is less [4, 5]. The problem of less biomass arises when biomass build-up reduces drastically as a result of the experimental parameters, for example, the extreme conditions of temperature, pressure, pH, etc. Despite the limitation of biomass quantity, an improved method of extraction of intracellular proteins from biomass as less as 5 mg (dry weight), without the need of liquid nitrogen based homogenization is described in this work. Various lysing methods and extraction buffers were evaluated to extract proteins using lyophilized biomass of *Aspergillus terreus* (Strain #A4636). The efficiency of the standardized method is demonstrated in terms of the quantity and quality of the extracted proteins as viewed on the SDS-polyacrylamide gel.

To check the efficiency of the standardized protocol, eight randomly chosen fungal isolates from eight different genera, isolated from different marine habitats, oyster mushroom, and one unidentified filamentous fungus were used. Fungal cultures were grown in modified Czapek Dox broth (CDB - sucrose replaced with 5% glucose and prepared in seawater) for 6-7 days at room temperature (28°C, static conditions), the biomass harvested, washed to remove residual seawater and media, lyophilized, and stored in airtight container till used for protein extraction. *Pleurotus ostreatus* was grown on Czapek Dox agar for 12 days at room temperature, the mycelia were harvested, and all the steps described above were followed.

It was reported that lyophilized biomass gave better protein profiles than wet biomass [2], hence lyophilized fungal biomass was used for entire analysis. To ascertain the best lysing condition, different methods of homogenization were tried such as sonication and vortexing with glass beads, mechanical disruption using FastPrep homogenizer (FastPrep®24, MP Biomedicals, USA) with lysing matrix A (MP Biomedicals, USA) and zirconium beads (D6006, Zymo Research, USA) (Table 1). Five hundred μ L of each extraction buffer and 5 μ L of 100 X Protease Inhibitor Cocktail (PIC, Serva #39104) were added to the lyophilized biomass for protein extraction individually. All the homogenizations using FastPrep homogenizer were performed at 6.5 m/s for 60 s. In case of single buffer extractions (S.No. 1, 2 and 3, Table 1), homogenization was performed twice with a rest time of 5 min, while for multi-buffer extractions (S. No. 4 and 5, Table 1), homogenization was performed twice for the first buffer (rest time of 5 min) followed by single homogenization for the remaining two buffers with the same biomass/pellet. The homogenized lysate tube was centrifuged at

14,000 rpm, 4°C for 15 min and the supernatant was collected. In case of multi-buffer extraction, supernatants were collected in a single tube.

Protein yield from sonicated biomass was very less (Table 1), as high-intensity sonication breaks the mycelia into smaller filaments but does not rupture the cell wall, thereby not releasing all the proteins [6]. Zirconium beads provided the best lysis of fungal mycelia, which reflected in the protein levels obtained (Table 1). Maximum yield of proteins with zirconium bead lysis could be due to their spiky-surface that aids in cell wall lysis; in addition, they are neutral in charge and higher in density (almost double as compared to glass beads of the same size) [7].

Constituents of extraction buffers in this study were selected based on their function. The Tris-HCl solution used in this method was chosen to maintain the ionic strength of the medium [8]. Detergents such as SDS and CHAPS control hydrophobic-hydrophilic interactions easing solubilization, while urea and thiourea increase the solubility of hydrophobic peptides by altering their structure [9]. The ratio between urea and thiourea varies typically from 9:0, 8:1, 7:2 to 6:3, as the combined strength of urea and thiourea in the buffer, should not exceed 9 M since at higher concentrations, urea and thiourea crystallize [10]. DTT is preferred since it is a di-thiol as compared to mono-thiol β -mercaptoethanol [11]. Magnesium chloride aids in stabilization of the extracted proteins [12].

Proteins obtained from first two methods (S.No. 1 and 2, Table 1) using TE and YBB buffers were too less ($> 2 \mu\text{g/mL}$). Tris-MgCl₂ 3-buffer system (Table 1, S. No. 5) yielded maximum proteins from lyophilized biomass of *A. terreus*, during standardization of the method. Fig. 1 is representing the quality of proteins obtained using different methods tried with lyophilized biomass of *A. terreus* #4636, with lane 6 showing the best protein profile. Tris-MgCl₂ 3-buffer system and FastPrep homogenization with zirconium beads were evaluated for its efficiency for protein extraction from different fungal isolates (Table 2). The method worked well for all the fungal cultures tested in this study, with the protein concentrations obtained above 1 mg/mL (Table 2).

It has been reported that the activity of proteases is very high in fungi, especially as mycelium ages [12]. When mycelium lyses, proteolysis starts dynamically, which could be stopped by addition of protease inhibitors and by working at low temperatures [1]. In this method, protease inhibitor cocktail (PIC) was added along with lysing buffer and lysed contents were intermittently cooled in ice (5 min) between homogenization pulses to neutralize the effect of proteases released during lysis. The intermittent cooling in ice also helped to nullify the heat generated during homogenization.

It was not possible to extract proteins from 5 mg biomass using conventional liquid nitrogen crushing in pestle and motor. Hence no relative quantity of protein extracted by liquid nitrogen crushing could be mentioned. Instead, comparison with previous studies of protein extractions using liquid nitrogen is reported in Table 3, which shows that recovery of proteins with respect to biomass employed using this method is quite high.

Quantification of the desalted protein extracts (done using BioSpin chromatography columns, BioRad, USA) was performed using Qubit Assay kit (Invitrogen, Q33211 USA) as per manufacturer's protocol. Desalting of proteins helps in removal of salts and other chemicals from the extraction buffers. The desalted protein extracts were electrophoresed using SDS-PAGE to check the quality and the yield of extracted proteins on 12 % resolving gel with 6 % stacking gel at a constant voltage of 90 V for one h and 45 min. Hundred μ g of protein extracts were loaded in each well. The gel was stained using silver staining method [13] (Fig. 2). The quality of the proteins obtained was good as evident from the SDS-polyacrylamide gel with distinct bands seen in almost all the lanes (Fig. 2).

The method of protein extraction described here is a rapid protocol (requiring around 20 min, excluding the time required for lyophilization) to obtain sufficient quantity of high-quality proteins. This method nullifies the disadvantage of biomass availability for protein extractions, thereby opening up avenues to study protein profiles of samples/organisms which are difficult to cultivate or sparsely obtained.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Contribution of authors

The idea was conceived by the corresponding author during the work on deep-sea fungal cultures, where obtaining biomass from fungi growing at elevated hydrostatic pressure is a challenge. The first author was involved in standardization of the method and drafting of the manuscript, and the work is part of her doctoral thesis to be submitted to Goa University. The second and third authors have validated the method by evaluating it for extraction from different fungal cultures. This is an original work by the authors and has not been published elsewhere either completely, in part, or in any other form. The manuscript has not been submitted to another journal. The results described here have been approved for publication by the responsible authority of the institute where the work was carried out, and all the persons entitled to authorship have been named.

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Legend to figures

Fig. 1 Mycelial protein profiles of *Aspergillus terreus* #A4636 obtained using FastPrep homogenizer lysing and different buffers

Lane 1: Protein molecular weight marker (PMW-Medium, Merck, India)

Lane 2 - YBB buffer, # A4636;

Lane 3 - YBB buffer, # MTCC479;

Lane 4 - Urea buffer, #A4636 (with SDS);

Lane 5 - Urea buffer, #A4636 (with IPG buffer);

Lane 6 - Tris-glycine combination buffer, #A4636;

Lane 7 - Standardized buffer, #A4636;

Lane 8 - Standardized buffer, #MTCC 479 (*A. terreus* strain);

Lane 9 - Urea buffer, #MTCC 479, (with IPG buffer);

Lane 10 - Tris-glycine combination buffer, #MTCC 479

Fig. 2 SDS-PAGE profile of proteins extracted from nine different fungal cultures using the optimized Tris-MgCl₂ buffer FastPrep homogenization with zirconia beads

Lane1: Protein molecular weight marker (PMW-Medium, Merck, India)

Lane 2: *Sarocladium strictum*

Lane 3: *Cladosporium cladosporioides*

Lane 4: *Hortaea werneckii*

Lane 5: *Acremonium alternatum*

Lane 6: *Purpureocillium lilacinum*

Lane 7: *Penicillium citrinum*

Lane 8: *Engyodontium* sp.

Lane 9: *Aspergillus flavus*

Lane 10: Unidentified filamentous fungal isolate