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A Comparison of Cell Wall Disruption Techniques for the Isolation of Intracellular Metabolites from *Pleurotus* and *Lepista* sp.

Rilka M. Taskova[a,b], Holger Zorn[a], Ulrich Krings[a], Henning Bouws[a], and Ralf G. Berger[a,*]

[a] Institut für Lebensmittelchemie, Universität Hannover, D-30453 Hannover, Germany. Fax: +49-511-762-4547. E-mail: rg.berger@lci.uni-hannover.de
[b] Institute of Botany, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

* Author for correspondence and reprint requests

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Different techniques were compared for their effectiveness in the disruption of the rigid cell walls of Basidiomycetes. Grinding under liquid nitrogen, stirred glass bead milling and enzymatic cell lysis were applied to the mycelia of *Pleurotus sapidus* and *Lepista irina* grown submerged. Each of the disruption procedures was evaluated by testing the quantity and quality of released intracellular metabolites: DNA, RNA, enzymes, and secondary metabolites. The most suitable method for nucleic acid isolation was grinding under liquid nitrogen, while bead mill homogenization was the superior technique for isolation of active enzymes. A new effective method is proposed for isolation of secondary metabolites with the aid of bead milling of fungal mycelia.

Key words: Basidiomycetes, Cell Disruption

Introduction

Basidiomycetes properties and wide range of application have led to a significant interest and intensive investigation of their systematics, ecology, genetics, molecular biology and biochemistry (Horner *et al.*, 1998; Wasser and Weis, 1999; Pointing, 2001). Disruption of fungal cells has appeared as the first critical step in molecular biology, enzymology and biochemical investigations. Several works have dealt with the comparison of different cell disintegration techniques for isolation of DNA from ascomycetous fungi (van Burik *et al.*, 1998; Haugland *et al.*, 1999; Kabir *et al.*, 2003). However, the cell disruption procedures suitable for Basidiomycetes have been scarcely studied. Their rigidity, specific cell wall composition and growth mode have been the major limitations for utilization of common procedures (Reader and Broda, 1988; Baldrian *et al.*, 1999; Taubert *et al.,* 2000).

Grinding under liquid nitrogen, glass bead milling and enzymatic cell lysis methods were applied to the mycelia of two Basidiomycetes, *Pleurotus sapidus* and *Lepista irina,* and monitored by evaluation of the quantity and quality of released intracellular metabolites: DNA, RNA, proteins and secondary metabolites. The aim was to assess the most suitable disintegration technique for each of the analytical approaches applied in fungal investigations, genomics, transcriptomics, proteomics, or metabolomics.

Experimental

Strains and cultures

The fungal strains, *Pleurotus sapidus* (DSMZ 8266) and *Lepista irina* (CBS 458.79), were grown submerged in a glucose-yeast extract broth (GYB; Onken and Berger, 1999). Homogenized mycelium was inoculated into 100 ml GYB in 300 ml Erlenmeyer flasks and incubated for 5 d at 24 °C and an agitation speed of 130 rpm. Mycelia were harvested by centrifuging at $1,000 \times g$ for 10 min at 4 °C (Varifuge 22 RS equipped with rotor HFA 22.50, Heraeus, Osterode, Germany).

Cell disruption

Grinding under liquid nitrogen

Aliquots of wet mycelia in 1.5 ml tubes were placed in a container with liquid nitrogen for 5 min. The frozen samples were transferred into a sterile pre-cooled mortar and ground under liquid nitrogen with a pestle. The buffer required for each of the test experiments [1.) potassium phosphate buffer (PPB), 50 mM, pH 7.4, for protein analysis or 2.) commercially supplied buffer for nucleic acid analysis] was added to the powder,

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mixed thoroughly and transferred to a microcentrifuge tube. Cell debris was pelleted by centrifugation at 15,800 × *g* for 5 min at room temperature using a 5415 C centrifuge (Eppendorf, Hamburg, Germany).

Bead mill homogenization

The container of a Dyno-Mill (Bachofen, Basel, Switzerland) was filled with 80 ml acid-washed 0.25 – 0.5 mm diameter glass beads (Roth, Karlsruhe, Germany) and 20 g wet mycelia. 30 ml buffer were added: 1.) Tris [tris(hydroxymethyl)aminomethane]-HCl buffer, 50 mM, pH 8, for nucleic acid analysis; 2.) PPB, 50 mM, pH 7.4, for protein analysis; or 3.) methanol for metabolic analysis. The samples were milled for 15 min at 4 °C at maximum speed. The glass beads and cell debris were pelleted by centrifugation at 2,000 × *g* for 5 min at 4 °C (Varifuge 22 RS equipped with rotor HFA 22.50, Heraeus) and aliquots corresponding to 100 and 300 mg of disintegrated mycelia were taken from the supernatant for analysis.

Enzymatic cell lysis

For the protein analysis, 100 mg wet mycelia were suspended in 2 ml buffer containing 0.05 M PPB (pH 7.4), 0.1% β-mercaptoethanol and ~ 300 U lyticase (Sigma, Steinheim, Germany). Samples were incubated at 30 °C for 30 min and then centrifuged at 300 × *g* for 5 min (5415 C, Eppendorf) to pellet spheroplasts. The supernatant was discarded by pipetting, replaced by 0.5% Triton X-100 solution and incubated for 15 min at 30 °C to lyse spheroplasts. Tubes were then centrifuged at 15,800 × *g* and the lysates transferred to a clean tube.

For the nucleic acid analyses, 100 mg wet mycelia were suspended in 2 ml buffer containing 1 M sorbitol, 0.1 M EDTA (pH 7.4), 0.1% β-mercaptoethanol and ~ 300 U lyticase. Samples were incubated at 30 °C for 60 min and centrifuged at 300 × *g* for 5 min (5415 C, Eppendorf). The supernatant was discarded by pipetting, replaced by the lytic buffer from the commercially supplied kit and spheroplast lysis followed the manufacturer's protocol.

Testing effectiveness of the cell disruption

Nucleic acid isolation and detection

DNA was extracted from 200 mg of disintegrated mycelia by a NucleoSpin Plant DNA purification kit (Macherey and Nagel, Düren, Germany) and RNA from 100 mg disintegrated mycelia using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The nucleic acid concentrations were quantified at 260 nm with a Lambda 12 UV/Vis Spectrometer (Perkin Elmer, Überlingen, Germany) and their quality was tested by an Agilent 2100 bioanalyzer (Agilent, USA) and PCR and RT-PCR analyses using standard procedures (Sambrook and Russell, 2001) and a laccase sequence (Linke *et al.*, 2005).

Determination of soluble protein

Water-soluble protein concentrations were determined by the method of Lowry *et al.* (1951) using the Bio-Rad D_C Protein Assay (Bio-Rad, Munich, Germany). Absorption was measured at 750 nm with a Lambda 12 UV/Vis Spectrometer (Perkin Elmer).

Enzyme activity assays

The enzyme activity of catalase (EC 1.11.1.6) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G-6-PD) was measured according to the methods described by Aebi (1983) and Deutsch (1983). Changes in absorption were monitored on a Lambda 12 UV/Vis Spectrometer and activity was calculated using UV-Winlab software (Perkin Elmer).

Metabolite profiling

The samples obtained by 24-h- and 5-day-extraction of mycelia with methanol and after bead mill homogenization were filtered, concentrated and purified on silica gel columns (Silica gel 60, 0.040 – 0.063 mm, Merck, Germany). Low-molecular-weight compounds were screened by HPLC (Jasco, Japan) with DAD (Jasco MD-910 multi wavelength detector, Jasco, Japan) and ELSD (Sedex 55, Sedere, Alfortville, France), LC/MS (Shimadzu LC-MS-QP 8000α system, Japan), and GC/MS (HP 5890 Series II gas chromatograph coupled to a HP quadrupole mass spectrometer 5989 A, Hewlett-Packard, USA).

Results and Discussion

Nucleic acid isolation

Samples of DNA in detectable amounts were obtained only by grinding the mycelia under liquid nitrogen (Table I). The RNA obtained after grinding under liquid nitrogen was of high integrity as proven by an Agilent 2100 bioanalyzer, PCR and RT-PCR analyses. Agarose gel electrophoresis

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Table I. Nucleic acid yield obtained by three disruption methods from *Pleurotus sapidus* and *Lepista irina*.

Species/method	DNA yield [μg g⁻¹]	RNA yield [μg g⁻¹]
Pleurotus sapidus		
Grinding under liquid nitrogen	9.1 ± 2.4	95.5 ± 31.8
Bead mill homogenization	–*	57.0 ± 25.4
Lyticase cell lysis	–	–
Lepista irina		
Grinding under liquid nitrogen	8.1 ± 1.6	109.0 ± 37.5
Bead mill homogenization	–	104.0 ± 11.3
Lyticase cell lysis	–	–

*No detectable amounts of DNA/RNA in the sample.

showed shearing of the RNA extracted by bead mill homogenization and the lyticase cell lysis method gave no detectable amounts of RNA. Grinding under liquid nitrogen appeared to be the superior method and lyticase cell lysis the least efficient for isolation of nucleic acid. Degradation of the isolated RNA after the bead milling process was most probably due to the specific parameters of the used Dyno-Mill which allowed disruption of high amounts of fungal material but did not provide an effective maintaining of RNA-free environment.

Protein isolation

The protein release was measured using the method of Lowry *et al.* (1951), and denaturation effects of the applied cell degradation methods were evaluated by monitoring the activity of catalase and glucose-6-phosphate dehydrogenase enzymes (Table II). Enzymatic cell lysis gave the least satisfactory results. As it was also expensive and time-consuming, it was considered as the inferior technique applied to protein isolation. Both, grinding under liquid nitrogen and bead mill homogenization, gave a significant overall yield of active intracellular enzymes. However, the fast and easy cell disruption by a Dyno-Mill was the preferable technique compared to the tedious procedure of grinding under liquid nitrogen, which does not allow the isolation of high amounts of protein.

Metabolite analysis

Methanolic extracts of *P. sapidus* and *L. irina* were obtained after 24 h and 5 days at room temperature and after bead mill homogenization (Table III). The samples were analyzed by high performance liquid chromatography (HPLC) with a combination of diode-array detector (DAD) and evaporative light-scattering detector (ELSD), liquid chromatography-mass spectrometry (LC/MS), and gas chromatography-mass spectrometry (GC/MS) and found to contain monosaccharides, sugar alcohols, fatty acids, sterols, ceramides, and terpenes. Glass bead milling in methanol showed lower yields compared to the simple extraction procedures (Table III) which could be explained

Table III. Yield (%)* of MeOH extracts from *Pleurotus sapidus* and *Lepista irina*.

	Pleurotus sapidus	*Lepista irina*
24-h-extraction	1.43	1.62
5-d-extraction	1.55	1.68
Bead mill extraction	1.37	1.37

* Yield (%), (weight of crude methanolic extract/wet weight of mycelia at start) × 100.

Table II. Protein yield and enzyme activity after grinding under liquid nitrogen, bead mill homogenization, and enzymatic cell lysis of mycelia of *P. sapidus* and *L. irina*.

Species/method	Protein yield	G-6-PD activity		Catalase activity*	
	[mg g⁻¹]	Total [U l⁻¹]	Specific [U g⁻¹]	Total [mK l⁻¹]	Specific [mK g⁻¹]
Pleurotus sapidus					
Grinding under liquid nitrogen	1.4 ± 0.2	90.0 ± 14.8	202.9 ± 66.6	80.3 ± 20.7	174.5 ± 16.3
Bead mill homogenization	1.6 ± 0.1	188.9 ± 16.7	174.6 ± 27.7	160.8 ± 23.0	148.0 ± 25.2
Lyticase cell lysis	0.8 ± 0.1	45.6 ± 0.4	91.6 ± 16.1	28.3 ± 22.1	61.0 ± 55.1
Lepista irina					
Grinding under liquid nitrogen	2.4 ± 0.01	119.5 ± 1.3	152.8 ± 1.1	661.5 ± 87.6	846 ± 114.6
Bead mill homogenization	1.7 ± 0.01	98.9 ± 4.3	88.5 ± 3.4	1589.8 ± 268.2	1424.0 ± 274.5
Lyticase cell lysis	0.7 ± 0.25	18.6 ± 1.9	39.9 ± 9.3	11.5 ± 3.4	24.0 ± 1.4

* K-rate constant used for catalase units since the abnormal kinetics of the enzyme (Aebi, 1983).

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by the comparatively difficult recovery of mycelia suspension from glass beads after the milling process. However, it led to the isolation of metabolites not present or present in small amounts in the simple fungal extracts. It is a fast, inexpensive technique, allowing disruption of sufficient amounts of mycelia and could be of interest as a new method for extraction of fungal metabolites.

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