EXTRACTION OF INTRACELLULAR CHITOSANOLYTIC AND CHITINOLYTIC ENZYMES FROM FILAMENTOUS FUNGI OF THE GENUS *Mucor*

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Abstract

Mucor is a genus of fungi belonging to the Mucorales, the most important order of zygospore-forming fungi. Mucor species are attracting growing interest due to their distinctive features, which make them excellent models for genetic studies, and also due to their wide industrial applications. They are a source of lipids, pigments, biosurfactants, and numerous enzymes, including proteases, lipases, cellulases, chitinases, and chitosanases. However, information regarding the isolation of intracellular chitosanolytic and chitinolytic enzymes from different Mucor species remains limited, and species-specific requirements for enzyme extraction have not been sufficiently investigated. In this context, the aim of this study was to develop a method for extracting intracellular proteins produced by Mucor racemosus CH52 and Mucor hiemalis CH80 and to confirm their activity in chitosan and chitin hydrolysis. Detergent lysis (Triton X-100, 0.5% w/v) was found to be the most effective method for isolating these enzymes from M. racemosus CH52 cells. In the case of the second strain, the best results were obtained by freezing and grinding biomass, combined with lysis using detergent (Triton X-100, 0.5% w/v). These methods enabled the production of cell-free extracts with high protein content and chitosanase and chitinase activities. The results demonstrated species-dependent differences in enzyme recovery and indicated the importance of developing tailored extraction strategies for individual Mucor species.

Keywords: *chitosanase, chitinase, intracellular enzymes, enzyme extraction, Mucor racemosus, Mucor hiemalis*

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1. Introduction

Fungi of the genus *Mucor* (order Mucorales, phylum Mucoromycota) comprise numerous fast-growing, saprotrophic species that are widely distributed in natural ecosystems. As a species-rich group of fungi, representatives of the genus *Mucor* are commonly found in soil, air, decaying plant material, food products, herbivore dung, insects, and the necromass of both plants and animals, particularly in moist environments. They play a crucial role in the decomposition of organic matter and nutrient cycling in ecosystems. Some *Mucor* species have a negative impact on humans because they can cause serious fungal infections, especially in immunocompromised individuals. However, others are used in food production and biotechnology due to their rapid growth and ability to produce valuable metabolites [1–3].

Mucor racemosus, along with *Mucor circinelloides*, is among the best-studied species in the genus. It is a dimorphic zygomycete that can grow rapidly in both aerobic and anaerobic environments. When exposed to air, it develops a dense grey mycelium that gradually darkens to brown or black due to the buildup of sporangia containing numerous dark sporangiospores [4]. *M. racemosus* belongs to the order Mucorales, a group of fungi generally considered thermotolerant. However, compared to other clinically relevant Mucorales, its maximum growth temperature is relatively low (it does not grow at temperatures above 34 °C). Although thermotolerance is an important virulence factor that enables fungal pathogens to colonise warm-blooded hosts, *M. racemosus* appears to have limited pathogenic potential. Nevertheless, it has been reported as an opportunistic pathogen, particularly in immunocompromised individuals [5–8].

Mucor hiemalis, in contrast, prefers cooler and moderate environments. Colonies are initially white and become grey or brown upon spore maturation, and their hyphae are similarly broad and coenocytic. This species rarely acts as an opportunistic pathogen and cannot grow at temperatures above 30°C [5, 9].

Strains of *Mucor* have attracted considerable interest in biotechnology due to their capacity to produce biopolymers such as chitin and chitosan, which have diverse applications in medicine, agriculture, and industry [9–11]. These fungi also secrete chitinolytic and chitosanolytic enzymes that can degrade chitinous substrates, facilitating the production of chito-oligosaccharides and other functional derivatives. Such enzymatic activities make *Mucor* strains valuable tools for bioconversion processes, including the enzymatic modification of biopolymers, synthesis of bioactive compounds, and sustainable valorisation of waste materials [12–15].

Biotransformation of chitin to chitosan is primarily catalysed by chitin deacetylases (EC 3.5.1.41), which enzymatically remove acetyl groups from *N*-acetyl-D-glucosamine residues in chitin, yielding the partially deacetylated polymer chitosan. In addition to deacetylases, chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132) are key hydrolases involved in the degradation of chitin and chitosan, respectively, by cleaving β-(1→4) glycosidic bonds in the polysaccharide chains [13, 16]. Enzymatic hydrolysis using chitinases and chitosanases is advantageous over chemical methods because it proceeds under mild conditions and allows better control over product size and structure, facilitating the production of defined chitooligosaccharides (COS) [13, 15].

Despite their biotechnological importance, research on intracellular chitosan- and chitinolytic enzymes produced by *Mucor* species remains limited. In particular, little is still known about effective methods for the isolation of these enzymes and species-specific factors influencing their recovery during extraction. Because fungal morphology and cell wall properties can influence the efficiency of cell disruption and enzyme recovery,

extraction protocols developed for one species may not necessarily be directly applicable to other members of the genus.

Based on previous studies on the *Mucor circinelloides* IBT-83 strain from the Institute of Molecular and Industrial Biotechnology of the Lodz University of Technology resources, this microorganism has been demonstrated to be an efficient source of mycelium-associated chitosanolytic enzymes, as well as chitosan derived from fungal biomass [12–14, 17]. These findings confirm the high biotechnological potential of the analysed strain. Therefore, over the years, studies have been conducted to characterise its metabolic and application properties. Currently, these studies are being continued and expanded to include two additional strains of the *Mucor* genus from the same collection, enabling comparative analyses and supporting the further development of biotechnological applications in this group of microorganisms. In this context, the aim of this study was to evaluate the effectiveness of selected methods for the isolation of intracellular chitosanolytic and chitinolytic enzymes produced by two strains of filamentous fungi: *Mucor racemosus* CH52 and *Mucor hiemalis* CH80. This study represents a preliminary step towards further isolation and characterisation of these enzymes.

2. Materials and Methods

2.1. Materials

The filamentous fungi *Mucor racemosus* CH52 and *Mucor hiemalis* CH80 were obtained from the resources of the Institute of Molecular and Industrial Biotechnology of Lodz University of Technology. The *M. racemosus* CH52 strain has previously been used in studies conducted at the former Institute of Technical Biochemistry (currently the Institute of Molecular and Industrial Biotechnology) [18–21]. Chitosan from shrimp shells was obtained from Heppe Medical Chitosan GmbH (Germany). The molecular weight and degree of deacetylation were $31 \cdot 10^4$ Da and ≥ 92.6%, respectively. Chitin from shrimp shells was obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals used in this study were of analytical grade.

2.2. Submerged Cultivation of Strains

Mucor strains were grown in Erlenmeyer flasks in a medium containing corn steep liquor (3.7% (w/v)) and rapeseed oil (2.7% (v/v)); the flasks were filled with 20% (v/v) medium. The sterile culture medium was inoculated with $6 \cdot 10^7$ sporangiospores (washed from 3-day-old slants containing brewing wort at a concentration of 8.5°Blg, pH 5.8) per 1 mL of culture medium. The strains were cultivated for 72 h at 30 °C with agitation at 180 rpm. The mycelia of the strains were harvested by vacuum filtration using Whatman filter paper (No. 1) and washed three times with distilled water to remove residual medium components. The biomass was then defatted by soaking in 96% (v/v) acetone at room temperature (22–25 °C) for 12 h with stirring. This step was performed to remove lipid constituents that could interfere with protein extraction and subsequent enzyme activity measurements. After decanting the solvent, the sample was air-dried at room temperature until a constant weight was achieved. The dried mycelium was subsequently stored under dry conditions until further analysis.

2.3. Protein Extraction From *Mucor* Strain Cells

2.3.1. Detergent-Based Lysis

A modified method described by Struszczyk *et al.* [12] was used to extract enzymes from *Mucor* strain cells. Defatted, dried biomass (1 g) was suspended in 10 mL of 50 mM

acetate buffer (pH 5.5), and one of the following detergents was added at concentrations of 0.1–1.0% (w/v): Triton X-100, Brij 35, Tween 80, or sodium cholate. The mixtures were stirred for 60 min at 4 °C and then centrifuged at 10,000 g for 10 min. The resulting supernatants (crude enzymatic extracts) were used to determine protein concentration and chitosan- and chitinolytic enzyme activity.

2.3.2. Physical Disruption and Detergent-Based Lysis

Physical methods of cell disruption were tested, including homogenisation (DI 25 basic IKA-Ultra Turrax homogeniser), freezing and grinding, and sonication (Bandelin Sonopuls HD 3100 homogeniser). Defatted and air-dried mycelium (1 g) was suspended in 10 mL of 50 mM acetate buffer (pH 5.5). The sample was optionally supplemented with Triton X-100 or Tween 80 (0.5% (w/v)). Extraction conditions were described in detail by Struszczyk *et al.* [12]. The obtained mixtures were centrifuged at 10,000 g for 10 min. The resulting supernatant (crude enzymatic extract) was used to determine the protein concentration and the activity of chitosan- and chitinolytic enzymes.

2.4. Determination of Enzymatic Activity

The 2 mL of reaction mixture contained 1 mL of 1% chitosan in 1% acetic acid or 1 mL of 1% colloidal chitin, 0.5 mL of acetate buffer (50 mM, pH 5.0) and 0.5 mL of crude enzymatic extract. The mixture was then incubated in a water bath shaker at 37 °C for 24 h. The reaction was stopped by 5 min of incubation in a boiling water bath. Control samples were first incubated for 5 minutes in a boiling water bath (to inactivate enzymes) and then for 24 h at 37 °C. All samples were centrifuged (10,000 g, 10 min) to remove precipitated chitosan or chitin. The level of reducing oligomers was quantified by the Somogyi-Nelson assay [22], using glucosamine or *N*-acetylglucosamine as reference standards. One unit of chitosanase or chitinase activity was defined as the amount of enzyme that catalysed the release of 1 μmol of D-glucosamine or *N*-acetyl-D-glucosamine per mL in 1 min. The colloidal chitin was prepared as described by Sauza *et al.* [23].

Enzymatic activity determined in crude extracts was expressed in U/ml. Specific activity was calculated by dividing the enzymatic activity by the protein concentration in the respective extract and expressed in U/g protein.

2.5. Protein Assay

Protein concentration was determined by the Lowry method [24] using bovine serum albumin as the standard.

2.6. Statistical Analysis

All experiments were conducted in triplicate. Statistical analyses were performed using Microsoft Excel 2013. Results are expressed as the mean ± standard deviation.

3. Results and Discussion

3.1. Effect of Detergent Lysis on the Release of Chitosanolytic and Chitinolytic Enzymes from *Mucor* Cells

Enzymes can be secreted by microorganisms into the culture medium during cultivation or remain inside the cells, which requires the use of various extraction methods, often time-consuming and expensive. Efficient extraction is essential to obtain high yields of active enzymes suitable for further purification and characterisation. Furthermore, the selected method should be economically feasible and suitable for large-scale applications.

Previous unpublished studies conducted with the investigated strains *Mucor racemosus* CH52 and *Mucor hiemalis* CH80 confirmed their ability to produce extracellular chitosanolytic and chitinolytic enzymes. However, the activities detected in the culture supernatants were relatively low under the applied cultivation conditions. In contrast, higher enzymatic activities were obtained from disrupted fungal biomass; therefore, the present study focused on the development of an extraction method and the characterisation of enzymes recovered from the mycelium.

This study evaluated the use of detergent lysis as an isolation method of chitosan- and chitinolytic enzymes from *M. racemosus* CH52 and *M. hiemalis* CH80 cells. Detergents are widely used to isolate membrane proteins by disrupting lipid bilayers and enabling their extraction for further purification and analysis [25, 26]. Four detergents: Triton X-100, Tween 80, Brij 35, and sodium cholate, were evaluated at various concentrations for their effectiveness in extracting enzymes. The tested compounds represented surfactants with diverse physicochemical properties, including nonionic detergents (Triton X-100, Brij 35, Tween 80) and an ionic bile salt-based detergent (sodium cholate), which may differentially affect membrane permeability and enzyme release. It should be emphasised that the experience gained from studies on the extraction and purification of enzymes from *M. circinelloides* was utilised [12].

The results of detergent-mediated cell wall lysis applied for the isolation of proteins with chitosanolytic and chitinolytic activity are summarised in Table 1. Among the detergents tested, Triton X-100 at a concentration of 0.5% (w/v) showed the highest efficiency for extracting proteins with the tested activity. For *M. racemosus* CH52, 49.28 ± 1.82 mg of total protein content was extracted. The measured chitosanolytic and chitinolytic activities were 8.03 ± 0.29 U/g and 2.97 ± 0.09 U/g, respectively. In the case of *M. hiemalis* CH80, these values were 22.15 ± 0.76 mg of protein, 3.42 ± 0.19 U/g and 1.64 ± 0.09 U/g, respectively. It should be noted that Tween 80 was the second most effective detergent among those tested, whereas sodium cholate exhibited the lowest enzyme extraction efficiency.

The results obtained are consistent with previous studies conducted with the *M. circinelloides* strain [12], which demonstrated that the mild nonionic detergent Triton X-100 was particularly effective at extracting intracellular chitosanolytic enzymes while maintaining their catalytic activity. In the study by Struszczyk *et al.* [12], Triton X-100 provided the highest chitosanolytic enzyme activity among the detergents tested. At the same time, significant differences were observed between the individual nonionic detergents; while Triton X-100 and Brij 35 demonstrated high extraction efficiency, Tween 80 was among the least effective detergents used in the study. It is worth noting that sodium cholate, an ionic detergent, also demonstrated relatively high enzyme extraction efficiency. This suggests that in this strain, enzyme release efficiency was not solely dependent on the ionic nature of the detergent.

3.2. The Effect of Combined Physical Disintegration and Detergent Lysis on the Extraction Efficiency of Chitosanolytic and Chitinolytic Enzymes

In subsequent experiments, mycelium homogenisation, freezing and grinding, and sonication were evaluated as common methods for the extraction of intracellular enzymes [27]. Physical methods achieve a high degree of cell disruption, but it should be remembered that their effectiveness depends on the microorganism species and process conditions [28]. In the presented experiments, these methods were additionally supplemented with previously selected lytic detergents, Triton X-100 or Tween 80, to enhance cell disruption and improve enzyme release efficiency.

Table 1. The effect of detergent type and its concentration on the extraction of chitosanolytic and chitinolytic enzymes from *Mucor* cells.

Detergent		***Mucor racemosus* CH52**			***Mucor hiemalis* CH80**		
Type	**Concentration [% (w/v)]**	**Protein [mg]**	**Specific activity [U/$g_{protein}$]**		**Protein [mg]**	**Specific activity [U/$g_{protein}$]**	
			chitosanolytic	**chitinolytic**		**chitosanolytic**	**chitinolytic**
Triton X-100	0.1	44.15 ± 1.14	6.95 ± 0.21	2.12 ± 0.10	16.36 ± 1.17	2.19 ± 0.16	1.20 ± 0.10
	0.5	**49.28 ± 1.82**	**8.03 ± 0.29**	**2.97 ± 0.09**	**22.15 ± 0.76**	**3.42 ± 0.19**	**1.64 ± 0.09**
	1	48.78 ± 1.57	7.15 ± 0.18	2.57 ± 0.07	19.64 ± 1.98	3.10 ± 0.21	1.45 ± 0.12
Tween 80	0.1	37.12 ± 0.97	4.18 ± 0.09	1.35 ± 0.09	14.73 ± 0.97	2.07 ± 0.16	1.25 ± 0.15
	0.5	**42.14 ± 1.26**	**6.47 ± 0.21**	**1.52 ± 0.11**	**17.42 ± 0.64**	**2.62 ± 0.14**	**1.31 ± 0.07**
	1	38.62 ± 1.13	5.72 ± 0.15	1.43 ± 0.09	15.06 ± 1.21	2.43 ± 0.14	1.19 ± 0.06
Brij 35	0.1	35.16 ± 1.59	3.07 ± 0.18	1.14 ± 0.08	12.56 ± 1.16	1.38 ± 0.16	0.90 ± 0.06
	0.5	37.28 ± 1.19	3.91 ± 0.27	1.24 ± 0.07	15.06 ± 0.41	1.95 ± 0.14	0.94 ± 0.08
	1	**40.56 ± 0.90**	**4.71 ± 0.17**	**1.35 ± 0.11**	**16.72 ± 0.35**	**1.13 ± 0.12**	**1.05 ± 0.09**
sodium cholate	0.1	30.29 ± 0.97	3.45 ± 0.16	0.98 ± 0.07	11.04 ± 0.28	1.11 ± 0.07	0.82 ± 0.08
	0.5	**34.28 ± 1.20**	**4.15 ± 0.11**	1.15 ± 0.09	**10.64 ± 0.69**	**1.37** ± 0.09	**0.90** ± 0.07
	1	32.15 ± 1.14	4.12 ± 0.15	1.19 ± 0.09	10.83 ± 0.63	1.21 ± 0.06	0.86 ± 0.09
Without detergent		14.16 ± 0.66	2.05 ± 0.09	0.91 ± 0.07	9.14 ± 0.29	0.71 ± 0.11	0.75 ± 0.06

Based on the obtained results (Table 2), it was concluded that the most effective method for extracting chitosan- and chitinolytic enzymes from *M. racemosus* CH52 cells was homogenisation of dried, defatted mycelium with the addition of Triton X-100 detergent (0.5% (w/v)). A cell-free extract containing 53.71 ± 1.27 mg of total protein was obtained, with chitosan- and chitinolytic activities of 9.55 ± 0.19 U/g and 3.18 ± 0.16 U/g, respectively. For the *M. hiemalis* CH80 strain, the most effective method was freezing and grinding in the presence of Triton X-100 (0.5% (w/v)). The resulting protein amount was 39.19 ± 0.28 mg, while the chitosanolytic and chitinolytic activities were 4.94 ± 0.12 U/g and 2.31 ± 0.10 U/g, respectively. It should be emphasised that the effect of applying an additional physical method was variable. In the case of *M. racemosus* CH52, no significant improvement in protein extraction efficiency or enzymatic activity was observed, suggesting that detergent lysis was already sufficiently effective. Similar results were obtained for the previously studied strain of *M. circinelloides* [12], where treatment of mycelia with Triton X-100 resulted in high protein extraction yields and the highest enzymatic activity. Furthermore, additional cell disruption methods, including homogenisation or freezing and grinding combined with detergent treatment, provided only modest improvements in enzyme recovery, suggesting that Triton X-100 alone was sufficient for efficient extraction. Although direct numerical comparison of protein yields and enzymatic activity values with those reported by Struszczyk *et al.* [12] is not possible due to methodological differences, the results obtained provide a basis for a comparative assessment of the applied extraction strategies.

For the second strain, the introduction of additional physical disintegration led to a significant increase in extraction efficiency - protein yield increased approximately twofold, while chitosan- and chitinolytic activities increased by 45% and 40%, respectively, compared to the method using only detergent. In this case, a significant portion of the enzymes could have been bound to more resistant cellular structures or located intracellularly, requiring more intensive disintegration.

According to the literature on chitinolytic and chitosanolytic enzymes in fungi, their localisation can be diverse, including secreted [29], cell-associated [12], and intracellular [30, 31] forms, the latter of which may require more aggressive extraction methods to ensure their complete release. Isolation of chitinases from the fungal *Rhizopus oligosporus* [30] or yeast *Candida albicans* [31] cells required mechanical disruption of the biomass, after which the enzymes could be effectively released from the cell extract and subjected to further chromatographic purification.

The lowest results obtained after sonication of mycelium may be due to the highly destructive nature of sonication, which involves cavitation and local temperature increases, leading to partial denaturation of enzymes and, consequently, reduced activity despite effective cell disintegration [27]. Similar results were reported for *M. circinelloides*, where sonication also resulted in the lowest enzyme activities compared with other extraction methods [12].

Compared with extracellular enzymes, cell-associated chitinases and chitosanases (intracellular or mycelium-bound) are much less frequently reported in the literature, limiting the available data for comparison. Furthermore, the lack of standardised methods for determining their activity, resulting from, among other factors, the use of different substrates, their concentrations, and reaction conditions, makes direct comparison of results between studies difficult.

It should also be emphasised that the literature on chitosan and chitinolytic enzymes produced by *M. racemosus* and *M. hiemalis* remains scarce. Alfanso *et al.* [32] studied the activity of chitosan- and chitin-degrading enzymes during the autolysis of fungi from

Table 2. The effect of combined cell disintegration methods on protein yield and enzymatic activity.

Method of protein extraction	***Mucor racemosus* CH52**			***Mucor hiemalis* CH80**		
	Protein [mg]	**Specific activity [U/$g_{protein}$]**		**Protein [mg]**	**Specific activity [U/$g_{protein}$]**	
		chitosanolytic	**chitinolytic**		**chitosanolytic**	**chitinolytic**
HOMOGENISATION						
with Triton X-100	**53.71 ± 1.27**	**9.55 ± 0.19**	**3.18 ± 0.16**	36.05 ± 0.41	4.63 ± 0.18	1.73 ± 0.05
with Tween 80	49.11 ± 1.29	7.17 ± 0.11	2.04 ± 0.12	35.18 ± 0.26	4.28 ± 0.16	1.79 ± 0.09
without detergent	40.42 ± 1.69	3.11 ± 0.10	1.35 ± 0.26	29.25 ± 0.21	3.31 ± 0.12	1.49 ± 0.06
FREEZING AND GRINDING						
with Triton X-100	51.18 ± 1.51	8.79 ± 0.27	2.71 ± 0.26	**39.19 ± 0.28**	**4.94 ± 0.12**	**2.31 ± 0.10**
with Tween 80	46.19 ± 0.98	8.06 ± 0.14	1.62 ± 0.21	33.81± 0.31	4.16 ± 0.09	2.56 ± 0.07
without detergent	39.19 ± 0.95	3.25 ± 0.21	0.91 ± 0.18	28.94± 0.27	3.67 ± 0.10	1.60 ± 0.09
SONICATION WITH ULTRASOUND						
frequencies 20 [kHz]	43.13± 1.84	2.12 ± 0.15	0.64 ± 0.11	29.18 ± 0.10	2.73 ± 0.11	1.13 ± 0.09

the order Mucorales (including *Mucor hiemalis*, *Mucor rouxii*, *Rhizopus stolonifer*, and *Syncephalastrum racemosum*). They analysed the enzymes present in the post-culture fluid to determine which hydrolases are involved in cell wall degradation. They found that chitosanase activity was present primarily in *M. hiemalis* and several other species. No exochitinase activity was observed in the studied strains, whereas endochitinase activity was observed in the late stages of autolysis (40-day cultures). The available data are based on the determination of enzymatic activity in cell extracts, without isolation and full characterisation of individual proteins. Therefore, these species remain an interesting area of research.

4. Conclusions

The study assessed the effectiveness of various cell disruption methods for isolating chitosanolytic and chitinolytic enzymes from *Mucor racemosus* CH52 and *Mucor hiemalis* CH80. The results indicate varying effectiveness of the procedures used depending on the strain. In the case of *M. racemosus* CH52, the combination of physical and detergent lysis did not significantly improve protein yield or enzymatic activity, suggesting that detergent lysis alone was sufficient to release the available enzyme fraction. It can be assumed that the enzymes were readily accessible, probably loosely associated with cellular structures. A different effect was observed with the second fungal strain, in which additional physical disruption significantly increased protein yield and enzymatic activity. This result indicates the presence of enzyme fractions that are more strongly bound to cellular structures or located intracellularly, requiring more intensive disruption. On this basis, it was found that the efficiency of isolating chitosanolytic and chitinolytic enzymes depends on the strain tested and the disintegration strategy used, which emphasises the need for the selection of extraction methods on an individual basis.

These findings provide new insights into species-dependent differences in the recovery of intracellular chitosanolytic and chitinolytic enzymes in *Mucor* spp. and demonstrate that extraction protocols effective for one species may not be directly transferable to others. This study contributes to the development of effective strategies for obtaining active intracellular enzyme preparations from filamentous fungi and may support future research on the biotechnological application of *Mucor*-derived chitosanolytic and chitinolytic enzymes. The results obtained represent the first step toward the purification and characterisation of intracellular enzymes from the investigated fungal strains.

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