

# ENDOPHYTIC FUNGI FROM *CHELIDONIUM MAJUS* L. AS A SOURCE OF CHITOSANOLYTIC ENZYMES: ISOLATION, IDENTIFICATION, AND ENZYMATIC ACTIVITY

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## Abstract

*Endophytic fungi are a promising source of enzymes with industrial potential. In this study, a preliminary evaluation of the capacity of endophytic fungi from *Chelidonium majus* L. to produce chitosanolytic enzymes was performed. Two strains, R and Z, were isolated from stem tissues and cultured on Czapek-Dox media. Microscopy classified strain Z within the genus *Aspergillus*. Chitosanolytic activity was evaluated in media supplemented with chitosan under both surface and submerged conditions. Both strains exhibited intracellular enzymatic activity, with the highest activity of 656  $\mu\text{mol}/(\text{min}\cdot\text{g})$ , determined for the strain labelled as Z. These results suggest that endophytic fungi may serve as a source of chitosanolytic enzymes. This is one of the first reports of such activity in endophytes from *C. majus* L., providing a basis for future studies.*

**Keywords:** fungal endophytes; *Chelidonium majus* L., chitosan; chitosanolytic enzyme

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## 1. Introduction

For many decades, fungi in plant tissues were primarily regarded as microorganisms responsible for causing various disease symptoms. Although plant microbial pathogens mainly reproduce inside the host, there is a group of microorganisms that inhabit the interior of plant tissues called endophytes, which refers to beneficial organisms that colonise the plant without causing disease [1]. Endophytic fungi not only do not induce disease symptoms in the plant host but also enhance its survival in adverse environmental conditions. Fungal endophytes have long been recognised as a significant source of biomolecules applicable across various industries, including bioremediation, agriculture, food production, and medicine [2, 3]. Numerous studies have shown that endophytic fungi can produce the same compounds as their plant host, like different alkaloids, flavonoids, steroids, and terpenoids [4–7]. Fungal endophytes and their metabolites confer protection to plants against various abiotic (e.g., drought or salt stress) and biotic factors (e.g., pathogenic microbes) [5].

Over the centuries, plants have evolved a robust immune system that protects them from detrimental pathogens, including fungi and insects. Pathogens and fungal endophytes possess microbe-associated molecular patterns (MAMPs), including chitin in the fungal cell wall, which is detected by plant receptors. Plants utilise specialised enzymes to decompose fungal cell walls and produce specific compounds that trigger chitin degradation [8, 9]. Nonetheless, nonpathogenic endophytic fungi are believed to provoke a minimal immune response, allowing them to endure within the plant [1, 10]. A strategy employed involves identifying the presence of foreign compounds, including chitin.

For example, an endophytic fungus, *Trichoderma atroviride*, sequesters chitin-derived GlcNAc and binds to chitin, thereby protecting against plant-produced chitinases and evading the host immune response [1, 11]. Similarly, the beneficial fungus *Epichloë festucae*, which transforms the chitin of its cell wall into chitosan through the action of chitin deacetylases, facilitates its transition from an epiphytic to an endophytic lifestyle in the leaf blades of *Lolium perenne* [1, 12].

Chitosan - a deacetylated derivative of chitin - is increasingly gaining attention for its biodegradability, biocompatibility, and antimicrobial properties, making it attractive to industries such as pharmaceuticals, agriculture, food, and cosmetics. Moreover, chitooligosaccharides obtained from chitin and chitosan show significant potential for applications as antibacterial agents, carriers for gene therapy, and in the field of wound healing [13]. However, these applications require the controlled conversion of chitin into products with specific degrees of polymerisation and acetylation levels. The application of enzymes generated by various groups of microorganisms, including chitin deacetylases (EC 3.5.1.41, ChDa), chitinases (EC 3.2.1.14), and chitosanases (EC 3.2.1.132), is increasingly important in this context [8].

In this context, endophytic fungi emerge as a compelling source of chitosanalytic enzymes. Their long-term symbiotic coexistence with plant hosts and adaptation to complex environmental niches have driven the evolution of diverse enzymatic systems, including those capable of modifying chitin and chitosan. These enzymatic tools contribute to immune evasion and successful colonisation and may also hold biotechnological value in the sustainable production of functionally tailored chitooligosaccharides and chitosan derivatives [9, 13].

This work presents preliminary results on the potential production of chitosanalytic enzymes by endophytic fungi isolated from the tissues of the herbaceous plant *Chelidonium majus* L. (greater celandine).

## **2. Materials and Methods**

### **2.1. Isolation of Fungal Endophytes**

The plant material *Chelidonium majus* L. was rinsed under running water, dried with a paper towel, and thereafter transferred to the laminar flow chamber. The material was segmented into separate parts, including root, stem, and leaf, each measuring 1 cm in size. In the next phase, all components underwent surface sterilisation, as described by Marchut-Mikolajczyk *et al.* [14]. Fragments of surface-sterilised plants were transferred to Czapek-Dox medium and incubated at 30°C for 5 days. The control sample comprised water from the last rinse of the material, which was placed on a clean substrate plate. The absence of growth following the incubation time demonstrated the efficacy of surface sterilisation of the plant material. After 7 days of cultivation, the obtained isolates were separated onto clean medium to obtain pure cultures and then subjected to microscopic analysis.

### **2.2. Surface Culture with Chitosan**

To assess the chitosanolytic properties of the isolates, a spot inoculation was performed using a loop in the centre of Czapek-Dox medium containing 0.2% (w/v) chitosan, derived from a 2% chitosan solution in 2% acetic acid (95% degree of deacetylation (DDA), viscosity of 1000 mPa·s Hepe Medical Chitosan GmbH (Germany)). The plates were incubated at 30°C for 5 days, and then the agar plate was flooded with an aqueous solution of 0.1% (w/v) Congo red for 20 min. Then the Congo red solution was decanted and decolourised with 1 M NaCl for 20 min to check the chitosanolytic activity. The appearance of a clearing zone signified substrate hydrolysis and enzymatic activity. All such zones were documented and measured.

### **2.3. Submerge Culture of Endophytic Isolates in the Presence of Chitosan**

Cultures were carried out in 500 mL flat-bottom flasks, using 50 mL of Czapek-Dox media: (a) clear, (b) supplemented with 0.1 g of chitosan, and (c) supplemented with 5 mL of a 2% chitosan solution. The media underwent sterilisation at 121°C for 15 min.

The flasks were inoculated with  $5 \cdot 10^7$  cells per 1 mL of medium, obtained from a 24-hour culture of the isolates in clear Czapek-Dox medium, and incubated on a shaker at 30°C and 160 rpm for 7 days. After cultivation, the mycelium was isolated from the liquid medium using a Büchner funnel, and the filtrate was centrifuged at 18,000 rpm and 4°C. Chitosanolytic activity was assessed in both the biomass and the filtrate. Prior to the enzyme activity assays, the biomass was dehydrated by grinding it in a mortar with acetone at a 1:5 ratio.

### **2.4. Chitosanolytic Activity**

An increase in the concentration of reducing sugars following chitosan hydrolysis was used to determine chitosanolytic activity, as described by Struszczyk-Świta *et al.* [15] and Struszczyk-Świta [16]. The reaction mixture comprised 1 mL of 2% chitosan in 2% acetic acid, 0.85 mL of 1 M sodium acetate, and 0.15 mL of enzyme solution (pH 5.5). An analogous reaction mixture containing 10 mg of enzyme was prepared to analyse chitosanolytic activity for biomass. Hydrolysis of chitosan was conducted at 37°C for 24 h, and it was terminated by boiling in a water bath for 5 min. Controls with the same composition as the samples were incubated in a scalding water bath for 5 min to inactivate the enzyme and subsequently incubated at 37°C for 24 h. A unit of chitosanolytic activity (U) corresponds to the amount of enzyme that, at 37°C and pH 5.5, releases 1  $\mu\text{mol}$  of reducing sugars from a 1% (w/v) chitosan solution within 1 minute [ $U = \mu\text{mol} \cdot \text{min}^{-1}$ ].

Chitosanalytic activity for biomass was calculated according to the formula:

$$\text{Activity} \left[ \frac{\mu\text{mol}}{\text{min} \cdot \text{g}} \right] = \frac{C_{\text{red}} \cdot V_p}{t \cdot m_e} \quad (1)$$

where:

$C_{\text{red}}$  – concentration of reducing sugars in the sample [ $\mu\text{mol}/\text{mL}$ ];

$V_p$  – volume of the sample in which the concentration of reducing sugars was measured [ $\text{mL}$ ];

$t$  – reaction time [ $\text{min}$ ];

$m_e$  – mass of enzyme used in the reaction [ $\text{g}$ ].

The chitosanalytic activity for the culture liquid was calculated according to the formula:

$$\text{Activity} \left[ \frac{\mu\text{mol}}{\text{min} \cdot \text{mL}} \right] = \frac{C_{\text{red}} \cdot V_p}{t \cdot V_e} \quad (2)$$

where:

$C_{\text{red}}$  – concentration of reducing sugars in the sample [ $\mu\text{mol}/\text{mL}$ ];

$V_p$  – volume of the sample in which the concentration of reducing sugars was measured [ $\text{mL}$ ];

$t$  – reaction time [ $\text{min}$ ];

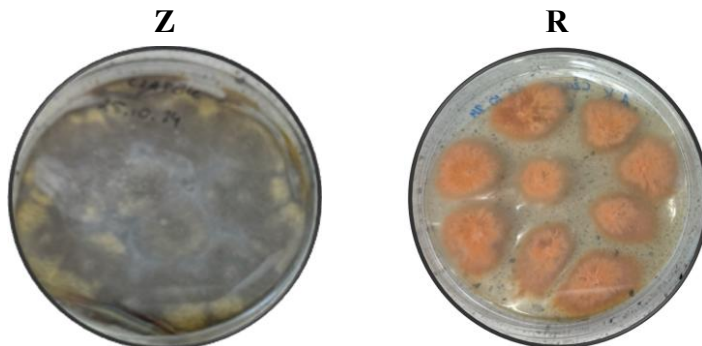
$V_e$  – enzyme volume used in the reaction [ $\text{mL}$ ].

All analyses were performed in triplicate, and the statistical analysis of the results was performed using the analysis of variance and Tukey's test in the R program.

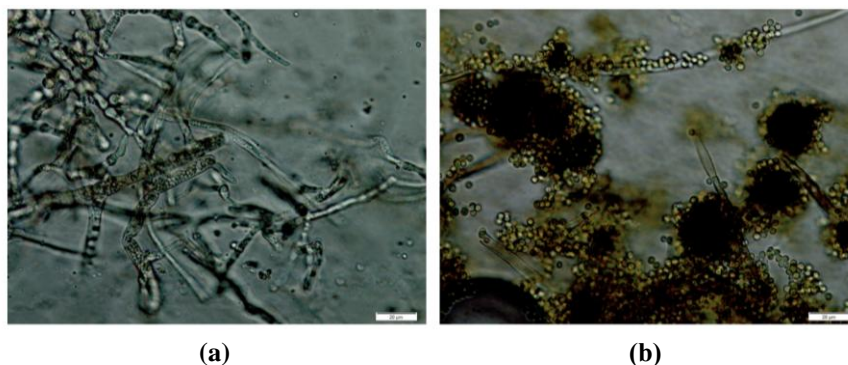
### 3. Results and Discussion

#### 3.1. Isolation of Endophytic Fungi from *Chelidonium majus* L. Tissues

*Chelidonium majus* L. plant material was surface sterilised under sterile conditions, and plant parts were transferred to a sterile Czapek-Dox medium and incubated at 30°C for 5 days. After the incubation period, the growth of two fungal cultures was observed. Both strains were grown from the stem of *Chelidonium majus* L. (greater celandine). Fungal isolates were not obtained from any other parts of the plant. Then, pure cultures of fungal endophytes were obtained, which were marked with the symbols Z and R (Figure 1). Subsequently, microscopic preparations of the isolates were conducted (Figure 2)



**Figure 1.** Endophytic fungi Z and R, isolated from the surface sterilised stem of *Chelidonium majus* L.

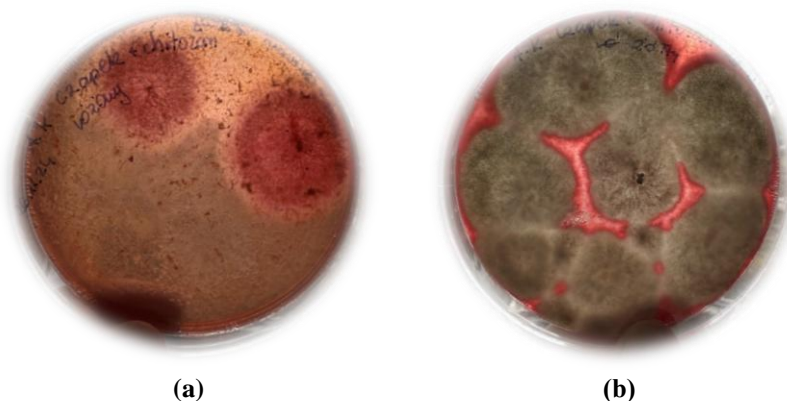


**Figure 2.** Microscopic preparation of (a) R endophytic isolate, (b) Z endophytic isolate.

Based on the morphology and microscopic images of the isolated fungal endophytes of the tested strains, the strain marked with the Z symbol was classified as an *Aspergillus* genus. Most of the literature on the isolation of endophytic microorganisms from *Chelidonium majus* L. pertains to endophytic bacteria [14, 17, 18]. The only group that successfully extracted eleven cultivated fungi from *Ch. majus* L. tissues was Huang and coworkers [19]. Of these, seven were recognised as *Colletotrichum*, and three were identified as *Fusarium* [19].

### 3.2. The Ability of Fungal Isolates to Produce Chitosanolytic Enzymes

To evaluate the ability of isolated endophytic fungi to synthesise chitosan-hydrolysing enzymes, surface culture was conducted on Czapek-Dox medium supplemented with chitosan. Following a 7-day incubation period, staining was performed using 0.1% Congo red. The staining results indicated the presence of 2 mm clean zones surrounding strains R and Z. Clear zones indicate that microorganisms can produce enzymes that decompose chitosan (Figure 3).



**Figure 3.** Cultures of endophytic fungal isolates on Czapek-Dox solid medium with chitosan: (a) clear zones obtained after Congo red staining for the strain marked with the symbol R, (b) clear zones obtained after Congo red staining for the strain marked with the symbol Z.

The potential of endophytic fungi for producing chitosanolytic enzymes is likely associated with their ecological function within plant tissues, where they release diverse

enzymes that promote colonisation and interaction with the host plant. Chitinases and chitosanases are important enzymes that facilitate the remodelling of plant cell walls and provide defence against pathogens, thereby enhancing the symbiotic relationship between the endophyte and its host [9, 20]. Reports about the synthesis of chitosanolytic enzymes by endophytic fungi are relatively scarce. However, literature data confirms that endophytic fungi are prospective sources of chitosanases. Rajulu *et al.* [20] found that endophytic fungi from the Western Ghats, India, synthesised chitosanases that were active on medium containing chitosan with differing levels of acetylation. Venkatachalam *et al.* [13] similarly discovered that endophytic fungi derived from marine algae and seagrasses exhibited considerable chitosanase activity, with 41% of the isolates demonstrating activity on chitosan with 56% acetylation and 66% on chitosan with 38% acetylation. These findings highlight the enzymatic adaptability of endophytic fungi across diverse ecological environments. Simultaneously, they affirm the capacity of endophytic fungi to synthesise a diverse array of enzymes, establishing them as significant assets for industrial applications.

### 3.3. Chitosanolytic Activity of Fungal Endophytes Isolated from *Chelidonium majus* L.

The activity of chitosanolytic enzymes from fungal endophytes isolated from *Chelidonium majus* L., named as R and Z, was examined under deep culture conditions. This analysis was carried out in pure Czapek-Dox medium and Czapek-Dox medium supplemented with 0.1 g of chitosan or 2% chitosan solution. Analyses were conducted on the post-culture liquid to evaluate the activity of extracellular enzymes and on the biomass ground from mortars to assess the activity of intracellular enzymes. The results are provided in Table 1.

For biomass-associated enzymes, 608  $\mu\text{mol}/(\text{min}\cdot\text{g})$  chitosanolytic activity for strain R was achieved solely after growth in a medium supplemented with a specific quantity of chitosan. Strain Z, initially classified within the genus *Aspergillus*, exhibited chitosanolytic activity in the biomass across all cultivation variants: 656  $\mu\text{mol}/(\text{min}\cdot\text{g})$  in pure Czapek-Dox medium, 600  $\mu\text{mol}/(\text{min}\cdot\text{g})$  in medium supplemented with a significant quantity of chitosan, and 526  $\mu\text{mol}/(\text{min}\cdot\text{g})$  in medium with chitosan solution. No extracellular chitosanolytic enzyme activity was observed for strain R. Nevertheless, all variations exhibited modest enzyme activity for strain Z, averaging around 0.104  $\mu\text{mol}/(\text{min}\cdot\text{mL})$ .

The results obtained are consistent with the available literature. *Aspergillus* species are known for their ability to produce chitosanolytic enzymes, particularly chitosanases. For example, *Aspergillus* sp. CJ22-326 has been studied for chitosanase production, with optimisation leading to a 3.92 U/mL activity in submerged fermentation [21]. Similarly, *Aspergillus* sp. QD-2 demonstrated chitosanase activity with optimal conditions at 55°C and pH 5.6 [22]. Researchers have also identified *Aspergillus fumigatus* strain 2T-2 for its high chitosanase production, indicating its potential in chitosan degradation applications. Notably, Yang *et al.* [23] isolated *A. fumigatus* strain 2T-2 from soil and optimised its fermentation conditions, achieving a chitosanase activity of 827.53 U/mL. The enzyme exhibited optimal activity at 55 - 60°C and pH 4.5, effectively degrading chitosan into chito-oligosaccharides and glucosamine salts, which are non-cytotoxic and hold potential for applications in food and agriculture industries.

**Table 1.** Chitosanolytic activity of endophytic fungi isolated from *Chelidonium majus* L. calculated from three independent replicates.

Endophytic fungi symbol	Enzyme source	Medium	Chitosanolytic activity [ $\mu\text{mol}/(\text{min}\cdot\text{g})$ ]
R	Biomass	Czapek-Dox	-
		Czapek-Dox with 2% chitosan solution	-
		Czapek-Dox with 0.1 g of chitosan	$608 \pm 3$
Z		Czapek-Dox	$656 \pm 3$
		Czapek-Dox with 2% chitosan solution	$526 \pm 5$
		Czapek-Dox with 0.1 g of chitosan	$600 \pm 4$
Endophytic fungi symbol	Enzyme source	Medium	Chitosanolytic activity [ $\mu\text{mol}/(\text{min}\cdot\text{mL})$ ]
R	Culture liquid	Czapek-Dox	-
		Czapek-Dox with 2% chitosan solution	-
		Czapek-Dox with 0.1 g of chitosan	-
Z		Czapek-Dox	$0.104 \pm 0.005$
		Czapek-Dox with 2% chitosan solution	-
		Czapek-Dox with 0.1 g of chitosan	-

*Note.* Chitosanolytic activity values are given as mean  $\pm$  standard deviation. ‘-’ denotes no extracellular chitosanolytic enzyme activity.

#### 4. Conclusions

This preliminary study demonstrates that two endophytic fungal strains, designated R and Z, were isolated from *Chelidonium majus* L. and found to produce chitosanolytic enzymes, primarily in the intracellular fraction. Strain Z, initially classified morphologically as *Aspergillus* sp., exhibited consistent enzymatic activity under all tested conditions, while strain R showed more limited potential. The predominance of intracellular activity suggests that the enzymes are retained largely within the fungal biomass. These findings highlight the potential of endophytic fungi as novel sources of biocatalysts for industrial applications. As an initial screening, the study provides a basis for further work on enzyme characterisation, including specificity, kinetics, and stability. Future research should also address molecular identification of the isolates, optimisation of culture conditions, and detailed biochemical analyses to better understand the regulation of intracellular enzyme production in fungal endophytes.

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