REVIEW

ENDOPHYTIC FUNGI AS CHITIN-MODIFYING ENZYMES PRODUCERS

Olga Marchut-Mikołajczyk

Institute of Molecular and Industrial Biotechnology, Faculty of Biotechnology and Food Sciences, Lodz University of Technology,
Stefanowskiego 2/22 Str., 90-537 Lodz, Poland
ORCID: 0000-0003-2041-3980
corresponding author: olga.marchut-mikolajczyk@p.lodz.pl

Abstract

Chitosan and chitooligosaccharides, which are products of chitin modification, have wide industrial applications. An improved understanding of the structure and properties of chitin-modifying enzymes has increased the interest in obtaining them from microbial sources and using them in the biotechnological production of these essential sugars. Endophytic fungi include a diverse group of microorganisms that inhabit the intercellular and intracellular areas of plant tissues, thus exerting beneficial effects on host species. They are considered an extremely valuable source of biologically active secondary metabolites and enzymes with high application potential. This review presents the potential of endophytic fungi to produce chitin-modifying enzymes and discusses the role of chitin modification by enzymes produced by fungal endophytes in their survival in the plant host.

Keywords: *endophytic fungi, chitin modification, enzymes*

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1. Introduction

Chitin, a linear homopolymer composed of *N*-acetylglucosamine linked by β-(1,4)-glycosidic bonds, is a prevalent biopolymer that is frequently encountered in the cell walls of fungi and the exoskeletons of crustaceans, invertebrates and insects [1]. Chitosan, which is a form of chitin that has undergone deacetylation, possesses antibacterial properties and is utilised in the management of plant diseases [2]. Both of these polymers possess non-toxic, biodegradable and biocompatible properties, making them suitable for many applications in the fields of food, cosmetics, agriculture and pharmaceuticals [3, 4]. Interestingly, chitooligosaccharides, which are oligomers produced from chitin or chitosan, exhibit substantial potential for application in wound healing, as antibacterial agents and as carriers in gene therapy [4].

To produce functional chitin derivatives, including chitooligosaccharides and chitosan, the original biopolymer must be modified. The enzymatic conversion of chitin into chitosan and chitooligosaccharides offers numerous benefits in comparison to chemical approaches [1, 5]. Chitin-modifying enzymes, such as chitin deacetylases (EC 3.5.1.41), chitinases (EC 3.2.1.14) and chitosanase (EC 3.2.1.132), can be used to produce products with the required molecular weight and degree of deacetylation [4, 6]. In this context, there is an increasing interest in chitin-modifying enzymes, which are produced by both bacteria and fungi. Considering the existing habitat and evolved mechanisms of adaptability, fungal endophytes appear to be a highly potential reservoir of novel enzymes, including chitin-modifying enzymes.

2. Endophytic Microorganisms

Endophytes are microorganisms, mainly bacteria or fungi, that inhabit plant tissues without causing any damage to the plant host. The term ‘endophyte’ was first used in literature in the nineteenth century. Endophytic microbes are primarily classified into two groups: facultative and obligatory. This classification is based on the way in which the microbes colonise the plant. While facultative endophytes do colonise plants at certain points in their life cycles, they can also live outside of plants at other points in their lives and establish a relationship with the soil in the host plants’ immediate rhizosphere. Obligate endophytes, on the other hand, are plant-dependent throughout their duration. Typically, they modify metabolic processes and plant products to ensure their own survival, or they reproduce between plant generations by means of vertical transmission and exploitation [7].

For many decades, researchers have been intensively studying the plant microbiome. It is now known that endophytic microorganisms benefit the plant by allowing it to survive in unfavourable environmental conditions, and they also serve as a valuable reservoir of bioactive compounds [8, 9].

2.1. Endophytic Fungi

Endophytic fungi are unique in that they only inhabit plant tissues, as opposed to mycorrhizal fungi that can grow into the rhizosphere and colonise plant roots [10]. Endophytic fungi have been the subject of extensive research due to their potential as a rich reservoir of novel biologically active compounds. These microorganisms have the ability to stimulate plant growth, work as biological protection agents and activate resistance to biotic and abiotic factors. Furthermore, certain endophytic fungi are capable of generating cytotoxic, antibacterial and anticancer compounds [7, 11–13].

These organisms can be divided based on their life cycle, plant hosts, phylogenetic features and ecological functions (Figure 1). The first group includes clavicipitaceous

fungal endophytes that reside in some grasses located in both warm and cool regions. Non-clavicipitaceous endophytic fungi are a distinct group that inhabit the tissues of non-vascular plants, ferns, conifers, and angiosperms. They are commonly classified within the Ascomycota or Basidiomycota families [8, 10, 14].

2.2.1. Clavicipitaceous Fungal Endophytes

Clavicipitaceous endophytes are frequently observed in the shoots of plants, particularly grasses that thrive in both cold and warm climates [7]. Typically, these microorganisms are related phylogenetically, and plants harbour a single genotype of the fungus. This category includes species that are symptomatic (type I), symptomatic-pathogenic (type II) and asymptomatic (type III) [15]. The colonisation of the plant by fungal endophytes from this particular group has the potential to enhance both drought resistance and biomass yield. Additionally, endophytes generate active chemicals that exhibit toxicity towards animals, perhaps imposing restrictions on herbivory. The specific impact is dependent on various elements, including the plant host species and prevailing environmental conditions [9]. Unfortunately, cultivating this category of fungal endophytes is very difficult, making it challenging to evaluate their potential for industrial application [7].

2.2.2. Non-Clavicipitaceous Fungal Endophytes

The ecological roles of non-clavicipitaceous endophytes, which are primarily ascomycetous fungi, are poorly understood despite their great diversity. This group of fungi can be isolated from almost all plants that thrive in terrestrial habitats under a variety of conditions, including extreme ones. Non-clavicipitaceous endophytes harbour an extensive variety of fungal species primarily classified within the ascomycetous group. Due to their extensive diversity, there is limited knowledge regarding their ecological functions. These endophytes exhibit a remarkable capacity to transition between endophytic and free-living lifestyles. The remarkable variety of this group of endophytes and the diverse ecological roles they carry out make these microorganisms the centre of attention of numerous research studies [9, 16, 17].

Non-clavicipitaceous fungal endophytes can be classified into three separate classes. This categorisation was established by considering the host colonisation method, the intergenerational transmission mechanism, the levels of plant diversity and the ecological significance. Class 1 endophytes possess the capacity to thrive in both above-ground and below-ground tissues, and the diversity of this class within individual host plants is generally limited [10, 18]. Conversely, class 2 and 3 endophytes are limited to the above-ground tissues and the roots, respectively. The host plant or tissue can harbour a significant variety of class 2 endophytes, with more than 20 species recorded from a single leaf tropical plant, as reported by Arnold et al. [18]. There has been insufficient research on the diversity of class 3 endophytes inside individual plants [10, 19].

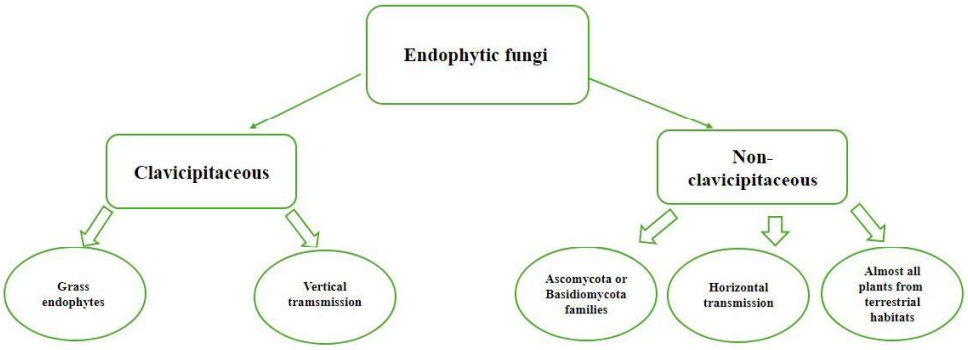

Figure 1. A descriptive scheme for endophytic fungi [10, 20].

3. Chitin-Modifying Enzymes Produced by Endophytic Fungi

Throughout the process of evolution, plants have developed an effective immune system that enables them to defend against harmful pathogens like fungi and insects. One of the key strategies involves detecting the presence of foreign compounds, such as chitin. With the help of specialised enzymes, plants have the ability to break down fungal cell walls and generate certain compounds that serve as triggers during the breakdown of chitin [21–23].

The environment in which endophytes live is not conducive to the growth of fungi due to the presence of tannins, phenols and other substances with biocidal activity, as well as the high salinity that often prevails in plant tissues. However, the ability of endophytic fungi to grow in these challenging conditions enables them to produce a wider variety and a greater number of enzymes. Moreover, compared with enzymes of the same classes produced by non-endophytic fungus, these enzymes are typically extracellular and show a spectrum of activity throughout a greater range of pH and salinity. However, the scientific literature on this topic is still quite limited [24].

3.1. Chitin Deacetylase

Despite the fact that fungal endophytes and pathogenic fungi share the same ecological niche, plant hosts exhibit distinct responses to infection caused by microorganisms from these two categories. To colonise plant tissue, endophytic fungi produce enzymes that facilitate the colonisation process, such as cellulases, laccases, pectinases and xylanases, which degrade the cell wall [25]. Nevertheless, this procedure activates the plant's immune responses. Thus, to survive in plants, endophytes require specific strategies to avoid detection by the plant's immune system. One such approach involves deacetylating chitin to chitosan to conceal its presence in the cell wall [26]. Chitin oligomers can be inactivated through various methods such as binding, degrading or deacetylating them. Of note, chitosan oligomers that have been completely deacetylated do not activate plant receptors, thus preventing any immune response [23, 26]. Research has indicated that the key enzymes responsible for the conversion of chitin into chitosan are chitin deacetylases (CDA) [23].

The first characterised CDA, namely PesCDA, was isolated from the endophytic fungus *Pestalotiopsis* sp. in 2016 [23]. The researchers identified the gene in *Pestalotiopsis* sp. that encodes CDA and then expressed it heterologously in *Escherichia coli*. Then, they examined the enzyme's substrate specificity and the chemical structure and biological activity of its reaction products. The study revealed that PesCDA is capable of modifying chitin oligomers, resulting in the formation of partially deacetylated chitosan oligomers with a distinct acetylation pattern: GlcNAc-GlcNAc-$(GlcN)_n$-GlcNAc ($n \geq 1$) [23]. Incubation of rice cells with the PesCDA products demonstrated that the chitosan oligomer products failed to induce an immune response. The findings suggest that the presence of endophytic CDA may prevent the plant's immune system from recognising the endophyte. The results further demonstrate that the use of recombinant CDA, which exhibits well-established regioselectivity in generating chitosan oligomers with distinct acetylation patterns, is a promising technique for the biotechnological synthesis of potentially effective bioactive compounds [23].

3.2. Chitinase

Mendrofa et al. [27] evaluated the ability of an endophytic fungus derived from the indigenous *Hedychium coronarium* J. Koenig to produce chitinase. Out of the 12 endophytic fungal isolates that were examined, only two strains

Aspergillus fumigatus JRE 4B and *Trichoderma afroharzianum* JRE 1A – demonstrated the capacity to generate extracellular chitinase (Table 1). Furthermore, the researchers analysed the antibacterial efficacy of the isolates against the pathogenic fungus *Fusarium oxysporum*. The tested pathogen led to growth inhibition in only two isolates, namely *T. afroharzianum* (> 70%) and *A. fumigatus* (> 30%), the same isolates that exhibited chitinase activity. The tested endophytic chitinases showed activity up to pH 4; however, at pH > 7, there was a significant decrease in activity. The chitinases from microbiological sources showed activity in the pH range of 5–8 [27]. The chitinase from *T. afroharzianum* JRE 1A was more stable than the chitinase from *A. fumigatus* JRE 4B. It retained > 80% activity in the pH range of 4–6, which indicates its tolerance to an acidic environment and its potential use in industrial processes operating at a low pH.

Depending on the chitinolytic strain, chitinase activity can be inhibited or stabilised by the presence of metal ions. Mendrofa et al. [27] also demonstrated that most tested metal ions slightly inhibited (>80%) the chitinases, especially the enzyme isolated from *A. fumigatus* JRE 4B. Conversely, the presence of various metal ions widely stimulated the activity of chitinase from *T. afroharzianum* JRE 1A, with only potassium (K^+) and calcium (Ca^{2+}) showing inhibition. Furthermore, the addition of manganese (Mn^{2+}) and zinc (Zn^{2+}) stimulated the activity of chitinase from both fungi, with Mn^{2+} providing the most pronounced stimulation. Additionally, other researchers have demonstrated that the crude chitinases derived from endophytes exhibited inhibitory effects on the growth of the phytopathogenic fungi *F. oxysporum* [28, 29].

Endophytic fungi belonging to the genus *Epichloë* (part of the Clavicipitaceae family) are symbionts of cool-season grasses and produce a variety of bioactive secondary metabolites that provide biotic stress resistance to the host. Noorifar et al. [28] documented the interactions between the endophytic *Epichloë festucae* and the perennial ryegrass (*Lolium perenne*). While the mechanisms by which *E. festucae* colonises ryegrass tissues are well understood, little is known about the means by which the fungus avoids inducing an immune response in the host through direct physical contact between its cell walls and the plant. Chitin is recognised as a significant constituent of the cell wall in *E. festucae* cultivated in axenic culture, but it is not present in the cell wall of endophytic hyphae, despite its evident presence in the septa [30–32]. *E. festucae* exhibits the capacity to reproduce both within plant tissue and as an epiphyte on the surface of grass leaves [30, 33]. Epiphytic hyphae originate from and remain connected to endophytic hyphae within the leaf. The composition of the cell wall of epiphytic hyphae undergoes changes during differentiation, resulting in the dominance of chitin within various divisions. These findings suggest that the chitin–chitosan substance in the cell wall of *E. festucae* undergoes changes when transitioning from free-living to endophytic hyphae and from endophytic to epiphytic hyphae. The genome analysis of *E. festucae* revealed the presence of three genes, *cdaA, cdaB and cdaC*, which encode proteins containing domains that are highly similar to CDA. These domains include a Zn-binding motif and catalytic-site amino acid residues that are essential for the activities of peptidoglycan and CDA. The data obtained from the study demonstrated that the conversion of cell wall chitin to chitosan, facilitated by chitin deacetylase, plays a vital role in sustaining the mutualistic symbiotic association between *E. festucae* and its grass host [30].

Malto et al. [32] investigated the capacity of three endophytic fungi derived from bamboo to break down chitin and to synthesise chitinases. Each of the three endophytic fungi demonstrated the capacity for growth on minimum media containing colloidal chitin as the sole carbon source. However, the strains *Aspergillus tubingensis* JB11 and *Daldinia eschscholzii* D12 had the fastest growth rate.

The investigated isolates demonstrated the capability to degrade chitin within 3–5 days of incubation. Although the three endophytic fungi exhibited similar levels of total chitinolytic activity (~0.35 U/ml), *A. tubingensis* JB11 showed the highest exochitinase activity (0.25 U/ml) [24, 27, 34]. The chitinase (GH18) genes of *A. tubingensis* JB11 and *D. eschscholzii* D12 were subjected to bioinformatic analysis. The analysis revealed variations in the GH18 chitinase sequences and the presence of additional domains. According to the scientists, a biochemical analysis of recombinantly produced chitinases is necessary to establish a connection between the chitinolytic activity of secreted fungal proteins and the GH18 genes found in these fungi. Furthermore, they suggest that the presence of sequence variation in the catalytic domain of GH18 chitinase with JB11 and D12 renders them very suitable as chitinase sources for biotechnological applications [24].

Table 1. Chitin-modifying enzymes produced by endophytic fungi.

Fungal endophyte	Host	Chitin-modifying enzyme	Activity	Reference
Aspergillus fumigatus JRE 4B	*Hedychium coronarium* J. Koenig	Chitinase	4.76 U/ml	[29]
Trichoderma afroharzianum JRE 1A		Chitinase	4.15 U/ml	
Pestalotiopsis sp.	Tea *(Camellia sinensis)*	Chitin deacetylase	n.d.	[23]
Daldinia eschscholzii D12	Bamboo (*Bambuseae* sp.)	Chitinase	0.35 U/ml	[24]
Aspergillus tubingensis JB11		Chitinase	0.38 U/ml	
Fomitopsis sp. JB10		Chitinase	0.35 U/ml	
Epichloë festucae	*Lolium perenne*	Chitin deacetylase	n.d.	[30]
Penicillium sp.	Seagrass *Cymodocea serrulata*	Chitinase	9 U/mg	[4]
Cladosporium sp.	Seagrass *Halophila ovalis*	Chitinase	5.9 U/mg	

Note. Abbreviation: n.d., not determined.

Venkatachalam et al. [4] conducted an interesting study. They examined the capacity of endophytic fungi isolated from seagrasses and algae to generate chitin-modifying enzymes. They obtained a total of 117 isolates, of which more than 14% exhibited chitinase activity, whereas nearly 40% displayed chitosanase activity. The isolates with the greatest chitinase activity were *Penicillium* sp. (9 U/mg) and *Cladosporium* sp. (5.9 U/mg). This work was the first to identify endophytic fungi of marine plants as a novel source of enzymes that alter chitin [4].

4. Conclusions

The demand for enzymes in various industrial processes remains significant. However, there is a need to establish techniques that produce enzymes with specified characteristics, such as long-lasting stability in terms of pH and temperature. Industrial applications also favour enzymes that demonstrate metal ion–independent activity and do not interfere with inhibitory compounds.

Because of their unique living conditions, endophytic fungi can serve as a valuable reservoir of enzymes with distinct features. The capacity to thrive in challenging environments such as high salinity and drought, along with the ability to utilise complex molecules as a carbon source, can lead to a wider range of enzymes being generated with increased stability. Although the ability of endophytic fungi to produce bioactive metabolites and new drugs with potential use in the environment, agriculture, medicine and the food industry has been explored widely, little attention has been paid to their use as a source of industrial enzymes.

Chitin and chitosanolytic enzymes have unique properties that make them useful to obtain new chitosan oligosaccharides with a specific structure and to biodegrade chitin waste, which brings benefits by minimising environmental contamination [35]. The knowledge of chitin-modifying enzymes produced by fungal endophytes remains limited. The predominant studies in this field have focused on understanding the endophyte–plant relationship and the significance of chitin-modifying enzymes in these interactions. It appears that chitin-modifying enzymes play a crucial role in facilitating symbiotic associations between fungal endophytes and plants [30]. The intriguing ability of endophytic fungi to use mechanisms that mask or transform chitin present in the cell wall into its derivatives, thereby facilitating compatible interactions with host plants, renders this taxonomic group a promising reservoir of enzymes capable of modifying chitin.

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