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# EFFECT OF CARBON SOURCE ON THE PRODUCTION OF LYTIC ENZYMES BY FILAMENTOUS FUNGI OF THE *TRICHODERMA* GENUS

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## ABSTRACT

The aim of this study was to evaluate the effect of cell wall biopolymers present in biomass of different types of filamentous fungi, yeasts and in biomass of insects on the expression of lytic enzymes in selected representatives of filamentous fungi: *Trichoderma harzianum* T33 and *Trichoderma citrinoviride* C1. Dried biomass of filamentous fungi, yeast and insect cells constituted a carbon source and also acted as an inductor of evaluated enzymes biosynthesis. The biosynthesis dynamics of chitinases, proteases and laminarinases depending on the type of inductor introduced into the culture were characterized and compared to the control cultures, not complemented with the cell wall biopolymers. The differences in the kinds and level of enzymatic activity, due to the addition of carbon source were demonstrated. The addition of insect biomass (1%) was the most preferred for expression of laminarinases and proteases in both *Trichoderma* strains (with a little predominance in the C1 strain). *Geotrichum* and *Botrytis* biomass was most effective in the induction of expressed chitinases (with a little predominance in T33 strain).

**Key words:** *Trichoderma*, chitinases, beta-1,3-glucanases, *Musca*.

## INTRODUCTION

The genus *Trichoderma* is one of the asexually reproducing filamentous fungi found in large quantities in almost all types of soils in moderate and tropical climate [12]. They have the ability to colonize the roots of plants (cultivated and wild) and the wooden parts of plants [12, 21]. Fungi of the genus *Trichoderma* have been known for 70 years to protect plants against phytopatogenic fungi such as: *Rhizoctonia*, *Sclerotinia*, *Sclerotium*, *Alternaria* or *Fusarium*, which is connected with the production of antibiotics against these microorganisms as well as the secretion of lytic enzymes (CWDE –*cell wall degrading* enzymes) and other factors. Therefore they are included in the category of BCAs (*Biological Control Agents*) [11]. Their rapid metabolism, the ability to produce an anti-microbial substances are key factors which contribute to the antagonism of these fungi against many pathogens [2]. Fungi of *Trichoderma* genus exhibit high capability to the production of hydrolases, such as chitinases, beta-glucanases and proteases which enable them to degradation the proteins and polysaccharides constituting the cell walls of filamentous fungi, especially pathogens. Lytic enzymes of *Trichoderma* also play an important role in their development, controlling physiological processes such as spore germination, growth ferns, hyphae growth, cell division, autolysis, nutrition [1, 8, 10]. Correlated effects of all factors, particularly on the enzymatic potential, significantly increase the antifungal activity of *Trichoderma* [7].

Chitinolytic enzymes produced by *Trichoderma* are members of family 18 including chitinases of bacterial, viral, animal and vegetable origin [13]. The attention needs to be drawn to endo-chitinase (E.C. 3.2.1.14) – cutting random bonds within the chain of chitin and produce low molecular weight: chitosan tetroses, chitosan trioses, diacetyl chitobioses to chitobiosidases (E.C. 3.2.1.29) – removing fragments of diacetylochitobioses from the non-reducing end of the chain and to beta-(1,4)-N-acetylo-glucosaminidases (E.C. 3.2.1.30) – consuming the products of endochitinases and chitobiosidases activity realizing monomers (N-acetylo-D-glucosamine) [5]. Among beta-1,3-glucanases produced by *Trichoderma* the two groups are

distinguished: endo-beta-1,3-glucanases (EC 3.2.1.58) and egzo-beta-1,3-glucanases (glucanohydrolases EC 3.2.1.6 and EC 3.2.1.39) [6, 20, 26].

In addition to the chitinases and beta-1,3-glucanases in the process of fungal cell wall degradation proteases are also involved, spreading matrix proteins and liberate – immersed beta-glucan and chitin [24].

*Trichoderma* lytic enzymes are induced by the appropriate substrates, which are a carbon and energy source.

Numerous studies have showed that not only laminarin or chitin, which are a good carbon source for the production of beta-1,3-glucanases and chitinases, but also purified cell walls of microorganisms or biomass containing these components induced enzymes biosynthesis [3, 4, 182]. Dry matter of cell wall of filamentous fungi and yeast contains 23–28% of beta-glucan and 8–11% of chitin [14]. In the insect biomass the chitin level could reach even 45% [14]. Therefore they could serve as an excellent source of carbon and energy as well as an inductor of biosynthesis of lytic enzymes in the cultures of *Trichoderma* fungi.

The presented research was based on the sterilized biomass of different filamentous fungi, particularly plant pathogens (*Alternaria*, *Aspergillus*, *Mucor*, *Rhizopus*, *Absidia* and *Botrytis)* and yeast (*Geotrichum*), as well as biomass from armor of insects (musca) in cultures of *Trichoderma* fungi. The influence of different biomasses on the process of beta-1,3-glucanases, chitinases and proteases biosynthesis was investigated. Such a procedure may be useful for the production of enzyme-based bio-against plant pathogens.

## MATERIALS AND METHODS

**Biological material.** The study involved two strains of *Trichoderma harzianum* T33 and *T. citrinoviride* C1 from the collection of the Department of Biotechnology and Food Microbiology (DBFM), Wrocław University of Environmental and Life Sciences. Those strains were cultivated in the presence of different inductors and the level of extracellular chitinases, beta-1,3-glucanases, and proteases was evaluated.

**The inductors biomass.** The dried biomass of the following strains *Alternaria* G0 (*Alternaria*), *Aspergillus niger* XP (*A. niger* XP), *Aspergillus niger* KB (*A. niger* KB), *Mucor hiemalis* (*M. hiem.*), *Rhizopus nigricans* 500 (*Rn*500), *Absidia glauca* 148 (*Abs. glauca*), *Botrytis cinerea* (*B. cinerea*), *Geotrichum candidum* OL (*G. cand.*) were used as carbon and energy sources and as inductor of lytic enzymes biosynthesis. All the strains were obtained from the DBFM collection. The strains were cultured in PBD (Potato Dextrose Broth) medium by shaking method (160 rpm.) at 25ºC for a period of 7 days, centrifuged, dried at 60ºC and milled.

***Trichoderma* culture conditions.** *T. harzianum* T33 and *T. citrinoviride* C1 cultures were carried out in Erlenmayer's flasks containing 50 ml of liquid PDB (Potato Dextrose Broth) medium (Difco) with the addition of carbon source (Tab. 1), by shaking method (160 rpm) at 25–28ºC for a period of 6 days. From each culture 2.0 ml of post-culture medium was obtained on the 2nd, 4th and 6th day of culture in order to analyze enzyme activities and carry out pH adjustment to 5.5.

Complemented cultures of both biomass of filamentous fungi (dry, milled) and musca biomass (dry, milled) as a supplement to the base PDB medium, where biomass biopolymers were also a source of carbon and inducer of lytic enzymes (glucanases, chitinases) were performed (Tab. 1). In order to evaluate the effects of inducing agents the culture with the addition of pure chitin was conducted as well as the control culture without additional inducer.

**Table 1. The culture variants**

| Culture | Type of inducer | Inducers biomass | Abbreviation |
|---|---|---|---|
| Induction culture | Cell wall biopolymers | *Alternaria* G0 (0.27%) | *Alternaria* |
| | | *Aspergillus niger* XP (0.27%) | *A. niger* XP |
| | | *Aspergillus niger* KB (0.27%) | KB |
| | | *Mucor hiemalis* (0.27%) | *M. hiem.* |
| | | *Rhizopus nigricans* 500 (0.27%) | *Rn*500 |
| | | *Geotrichum candidum* OL (0.27%) | *G. cand.* |
| | | *Absidia glauca* 148 (0.27%) | *Ab. gl* |
| | | *Botrytis cinerea* (0.27%) | *B. cin* |
| | | *Musca* (0.27%) | m 0.27% |
| | | *Musca* (1.0%) | m 1.0% |
| | Chitin | Chitin (0.27%) | ch 0.27% |
| | | Chitin (1.0%) | ch 1.0% |
| Control culture | Without inducer | | PDB |

**Analytical methods.** Activity of beta-1,3-glucanases (or chitinases) were expressed in nKat / ml of glucose released (or glucosamine) from laminarin substrate (or colloidal chitin), performing the reaction at 50C at pH 4.8 for 30 min (beta-1,3-glucanases) or 1h (chitinases). Reducing sugars released in the reactions were determined by the DNS method [27].

The proteolytic activity was measurement by a modified Anson method, using casein as substrate at pH=6,0 and 30ºC. One unit (JP) of proteolytic activity was expressed as 0.1 increase in absorbance at 280 nm per minute [16].

## RESULTS AND DISCUSSION

In the presented research, a number of culture efforts was conducted to demonstrate the effects of biomass of fungi and insects rich in chitin and beta-glucan on chitinases, beta-1,3-glucanases and proteases synthesized by two species of filamentous fungi *T. harzianum* T33 and *T. citrinoviride* C1. The chitin and beta-glucan contained in the biomass of fungi, and insects, were used as the carbon source and inductor of enzyme biosynthesis. The results were compared with control cultures without inductors addition.

The strain *T. harzianum* T33 was generally characterized by much higher efficiency in the biosynthesis of extracellular chitinases (0.5–2.4 nKat/ml) as compared to strain C1 (0.1–0.5 nKat/ml) (Figs 1 and 2) on all tested carbon sources. However, in the medium with the addition of an insect biomass or powdered chitin, laminarinases were produced more efficiently by the *T. citrinoviride* C1 strain rather than *T. harzianum* T33 strain (Figs 3 and 4).

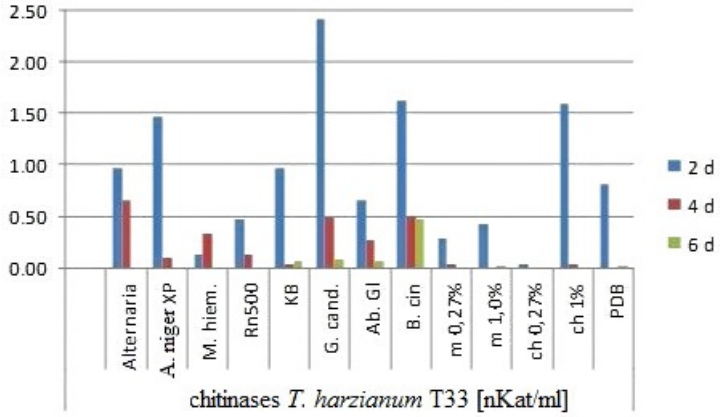


**Fig. 1. The dynamics of the chitinases biosynthesis by *Trichoderma harzianum* T33**

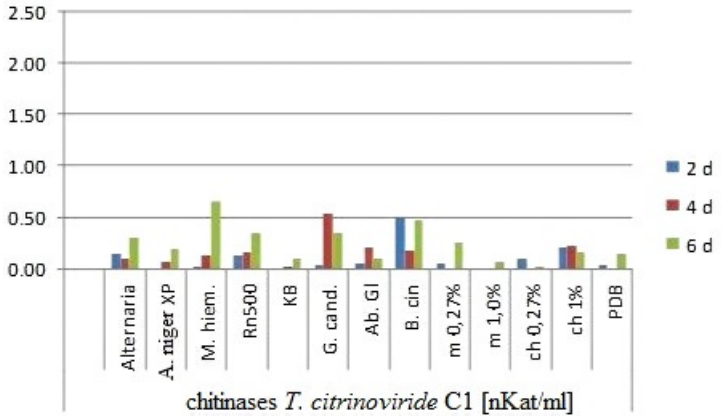


**Fig. 2. The dynamics of the chitinases biosynthesis by *Trichoderma citrinoviride* C1**

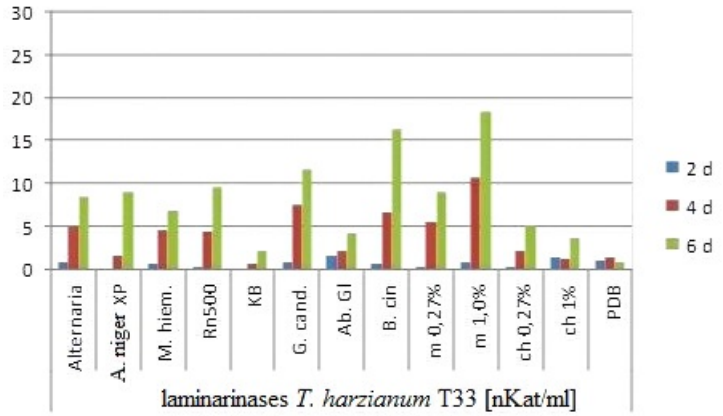


**Fig. 3. The dynamics of the β-1,3-glucanases biosynthesis by *Trichoderma harzianum* T33**

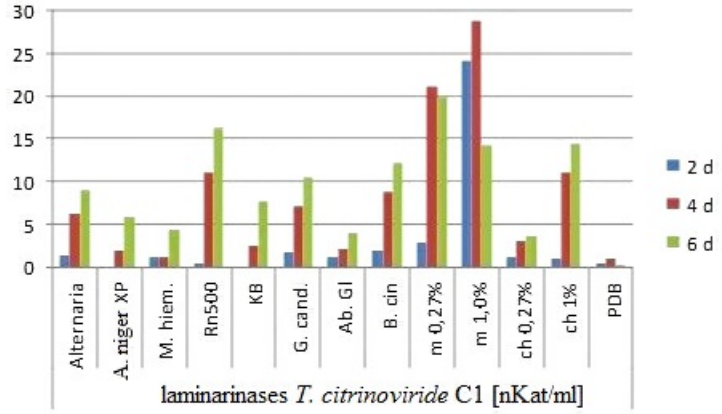


**Fig. 4. The dynamics of the β-1,3-glucanases biosynthesis by *Trichoderma citrinoviride* C1**

The most effective inducer of chitinases in cultures of strain T33 turned out to be the biomass biopolymers from *Geotrichum candidum*, which was revealed by the presence of the highest enzymatic activity (2.41 nKat/mL). In the presence of powdered chitin activity was 1.5 nKat/ml only, but in control (PDB) – 0.75 nKat/ml. This effect might have been caused not only by a more condensed structure of the substrates but also other components present within fungi and yeasts biomass.

Biosynthesis of laminarinases by both tested strains was characterized to be of up 10 times higher levels of activity (up to 28 U/ml) than chitinases (Figs 3 and 4). Both strains released the extracellular enzymes throughout the whole culture, in most cases reaching the maximum activity on the 6th or the 4th day, as in the case of *T. citrinoviride* C1 culture in the presence of insect biomass.

Insect biomass biopolymers were the most effective inducers of the biosynthesis of laminarinases for both strains: *T. citrinoviride* C1 (28 nKat/mL) and for *T. harzianum* T33 strain (18 nKat/mL). The greater level of laminarinases activity in the presence of various inducers for the control culture (PDB) may indicate an inducible enzyme biosynthesis in both strains. Stimulation of these strains for the laminarinases synthesis proceeded much more efficiently under the influence of insect biomass than under the influence of the filamentous fungi or yeast biomass, suggesting a positive impact of other components of the insect biomass on the laminarinases biosynthesis.

In the performed cultures the activity of extracellular proteases, as enzymes often associated with chitinases and beta-glucanases was evaluated. High diversity of their activity, depending not only on the strain but also on the overall composition of the medium was revealed (Figs 5 and 6). Generally of *T. citrinoviride* C1 strain synthesized proteases at a higher level (range 2.0 to 11.4 JP) than *T. harzianum* T33 (from 2.2 to 9.8 JP), but in the case of the control cultures of both strains determined activity of these enzymes was at the same level, which may be indicative of their constitutive synthesis mechanism.

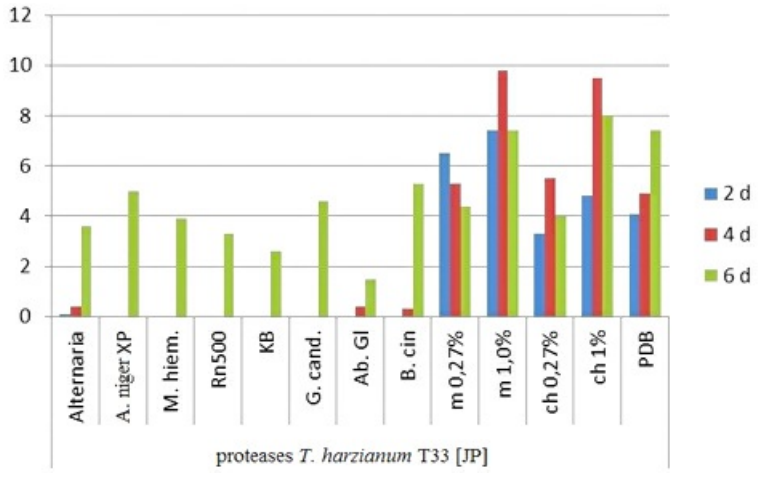


**Fig. 5. The dynamics of the proteases biosynthesis by *Trichoderma harzianum* T33**

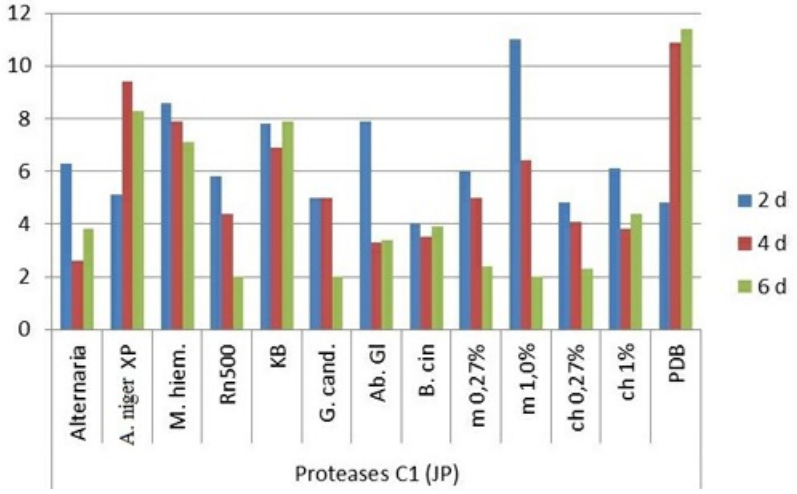


**Fig. 6. The dynamics of the proteases biosynthesis by *Trichoderma citrinoviride* C1**

The scientific interest in *Trichoderma* genus is mainly the result of their ability to produce numerous lytic enzymes (such as chitinases, beta-glucanases or proteases), and the extraordinary adaptability to adverse environmental conditions combined with the utilization of a wide variety of compounds used as the source of carbon [2, 21]. The focus of this study was to determine the effect of inactivated microbial or insect biomass containing chitin and beta-glucans in the induction of chitinases and beta-glucanases expression in the cultures of the two strains of the genus *Trichoderma: T. harzianum* T33 and *T. citrinoviride* C1.

Selection of these two strains of *Trichoderma* was associated with the results of earlier studies [23]. The interactions between strains of *Trichoderma* (e.g. *T. harzianum* T33, *T. citrinoviride* C1) and pathogenic strains of filamentous fungi (such as *Botrytis cinerea, Fusarium oxysporum, Fusarium poae*) were investigated by performing two-strains biotic tests. The obtained results allowed to establish that the strain with the strongest antagonistic properties was *T. harzianum* T33, a slightly lower percentage in inhibiting the growth of phytopathogens showed *T. citrinoviride* C1. The *Trichoderma* sp. moulds are known for its antagonistic properties with respect to the above-mentioned pathogens [12].

This effect may be due to their ability to produce over 100 metabolites with antibiotic activity, but also by biosynthesis of enzymes capable of degrading chitin and beta-glucans, i.e. compounds which are a part of the fungal cell wall. In consideration of the above characteristics of filamentous fungi of this genus intensive studies are carried out on their application in agro-ecological (biocontrol) [http://trichoderma.iwarz.pl], [6, 7] and industry [21].

The research focused on assessing the effective production of the lytic enzymes in shake flasks. The differences in ability of tested strains for the production of extracellular hydrolases were demonstrated and depended on the type of added carbon source, acting also as inducers, as well as on the strain-producer of these enzymes.

In the literature, it is noted that there is a significant influence of the complementary of culture compounds inducing the synthesis of a specific group of enzymes, such as a positive effect of chitin to synthetize chitinases and laminarin to synthesize beta-1,3-glucanases [28]. There is also pointed out that the addition of pathogen biomass in *Trichoderma* cultures, resulted in the achievement of comparable activity on a culture with addition chitin or laminarin. According to Noronha et al. [20], the source of biomass is also important, as beta-glucanases activity obtained in the presence of *Pythium* sp. were even two times higher than in the presence of *S. rolfsii* biomass and laminarin. In the studies of Witkowska et al. [28] a positive effect of *Fusarium* biomass on the production of chitinases and beta-glucanases in cultures of *T. citrinoviride* strain C1 was shown. Chitinases and glucanases reached levels of 1.17 nKat / ml and 28 nKat / ml respectively, whereas in the present study *T. citrinoviride* C1 strain chitinases activity level of 0.75 nKat / ml were reached in the culture of the *M. hiemalis* biomass. According to the research of Vazquez-Garciduenas et al. [26] the cell walls biomass of various pathogens added to the medium as the carbon source and enzyme inducer also may have varying effects on the level of expression of enzymes (particularly laminarinases) even due to the small differences in the construction of these walls.

The research performed by Nampoothiri et al. [19] showed that the technical chitin flakes only slightly affected the increase in the biosynthesis of chitinases in comparison to the control (no additives). They obtained better results when added to cultures the same quantity of chitin, but in a colloidal form. It might be associated with the availability of chitin, which was in the form of colloid is easily absorbed and degraded. In our study, the addition of 1.0% of native fragmented chitin in *Trichoderma* cultures, had a positive effect on the level of expression not only of chitinases, but also laminarinases and proteases in examined strains. Studies on the structure of chitin shells of insects present in the example of chitin isolated from bumblebee (*Bombus terrestris*) were carried out by Majtán et al. [17]. They compared the structure and chemical composition of the chitin obtained from the armor of bumblebee and chitin armor isolated from marine shrimp, reaching the conclusion that the insect chitin with a better organized structure, a lower degree of acetylation and containing less minerals in comparison to the chitin of shellfish armor is more accessible enzyme-substrate. In our study, in cultures of *Trichoderma* sp., biopolymers shell insects of the genus *Musca* proved to be the best inducer of laminarinases and proteases expression.

The relationships resulting from the difference between the strains as well as medium supplementation with various source of

carbon were demonstrated by Shakeri and Foster [24]. They performed a number of cultures of two strains of*Trichoderma harzianum* in minimal medium complemented with interchangeably carbon sources such as glucose, chitin, biomass *B. cinerea* or casein towards the biosynthesis of chitinases and proteases. They discovered that the biomass of pathogenic fungus *B. cinerea* was an excellent inducer of the biosynthesis of both chitinases and proteases of *Trichoderma* strains. In our study the ability of test strains of filamentous fungi to synthesize the extracellular proteases in the presence of biomass of fungi, yeast, insect biomass as well and in shacked cultures was also demonstrated.

Fellow researchers emphasize the fact that the lytic enzymes produced by the fungi of the *Trichoderma* genus are more active and exhibit more effectiveness against pathogens than enzymes produced by other microorganisms and plants [15]. For this reason, they are widely used in industry, agriculture, and environmental protection (bio-control, food and fodder production, Single Cell Protein degradation of chitin-rich waste [22]. Such a wide range of applications bears the need to develop effective and economic methods of obtaining the above-mentioned enzymes. One such method may be a culture of microorganisms (inter alia, filamentous fungi of the genus *Trichoderma*) able to produce them in the presence of biomass of plant pathogens, as a carbon source. This method could be obtained of biopreparation on the basis of*Trichoderma* lytic enzymes useful as antagonists against plant pathogens.

## CONCLUSION

Strain *T. harzianum* T33 was more effective in the biosynthesis of chitinases, than strain *T. citrinoviride* C1 in laminarinases and protease biosynthesis.

The complementation of the culture by musca biomass was more effective in the expression of the biosynthesis laminarinases as well as protease in case of both tested strains with a predominance of C1 on the T33.

The addition of fungi biomass (*Botritis*) or yeast (*Geotrichum*) to the culture particularly favored chitinases biosynthesis of both strains, with predominance of the T33 strain on the C1.

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