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Effect of Lyophilization on Survivability and Growth Kinetic of *Trichoderma* Strains Preserved on Various Agriculture By-Products

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Abstract

Growth kinetics of four *Trichoderma* strains was tested on lignocellulosic by-products in solid state fermentation (SSF). The strains were also analyzed for their survival rate and growth after lyophilization on these carriers. All applied monocomponent and bicomponent media were substrates for the production and preservation of *Trichoderma* biomass. However, the maximum number of colony forming units (CFU/g dm) was acquired on bicomponent media based on dried grass and beet pulp or grass with corn cobs, when compared to monocomponent media. Although the process of lyophilization reduced the survival rate by 50–60%, the actual number of viable cells in obtained biopreparations remained relatively high ($0.58 \times 10^8 – 1.68 \times 10^8$ CFU/g dm). The studied strains in the preserved biopreparations were characterized by a high growth rate, as evaluated in microcultures using the Bioscreen C system.

K e y w o r d s: *Trichoderma* sp., Bioscreen C system, lignocellulosic carriers, lsurvivability after lyophilization

Introduction

Due to the growing awareness of the society with respect to environmental protection and increased concern about health, alternatives to chemical methods of plant protection are sought. Hope is seen in biological methods based on natural agents that inhibit the development of undesirable microflora (Gerhardson, 2002; Manso *et al.*, 2010). The search for isolates with effective protective properties, mainly against phytopathogens, is based on testing their antagonistic action, identification of promising strains, and their subsequent use in the production of biologicals. Fungi of the *Trichoderma* genus are often used as biological agents in biocontrol products. Fungi of this genus exhibit many advantageous properties, that allow their application in plant protection (Błaszczyk *et al.*, 2014; Smolińska *et al.*, 2014). The most desirable features include: widespread occurrence in the soil environment, dynamic growth, abundant sporulation, rapid colonization of the roots of plants as well as the ability to utilize a wide variety of nutrients from the soil (Jash and Pan, 2007). In addition, these fungi are producers of various hydro-lytic enzymes, including cell wall degrading enzymes (CWDEs), which damage cell walls of phytopathogens, such as chitinases, beta-glucanases or proteases. They also synthesize numerous fungitoxic compounds, such as peptaibole, sesquiterpenoids, polyketides, isonitriles and certain octaketide antibiotics, and demonstrate the ability of mycoparasitism and induction of systemic plant resistance (Viterbo *et al.*, 2002; Howell, 2003; Daniel and Filho, 2007; Degenkolb *et al.*, 2008). The result of complex action of antagonistic agents of *Trichoderma* fungi is growth inhibition of phytopathogens from genera *Rhizoctonia*, *Phytium*, *Sclerotium*, *Fusarium*, *Botrytis* and *Verticillum* (Witkowska and Maj, 2002; Monte and Llobell, 2003; Matroudi *et al.*, 2009; Piegza *et al.*, 2009; John *et al.*, 2010; Monteiro *et al.*, 2010). Furthermore, these fungi are resistant to their own metabolites, metabolites produced by other microorganisms, terpenoid phytoalexins secreted by plants, as well as fungicides and heavy metals present in the soil (Khan and Shahzad, 2007).

A suitable method for the preparation of products based on *Trichoderma* fungal cultures are cultures in solid medium, such as solid state fermentation (SSF)

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with the use of lignocellulosic waste materials. These compounds are inexpensive, readily available in large amount sand, generally do not require nutrient supplementation. Moreover, these materials are rich sources of C, N and P, which contributes to rapid growth and sporulation of fungi. One of the main advantages of the SSF culture techniques is low water consumption, and thus the limited production of wastewater and minimization of energy consumption (Thomas *et al.*, 2014; Mondala, 2015).

Industrial biopreparations containing microorganisms should demonstrate a high rate of survival, growth and maintenance of important biological properties after preservation of biomass, as well as the stability of these characteristics during storage. One of the methods for the preservation of microbial biomass is lyophilization. However, the application of the large difference of temperatures in the process of lyophilization can lead either to the destruction or deformation of the cell membrane, as well as denaturation of cellular proteins. Therefore, protective agents are typically used, which should be selected individually for each microorganism. The preservation of biomass in the process of lyophilization allows for the long-term storage of microbial cells, but also draws attention to a large variation in survival rate that depends on factors, such as the preserved strain, the stage of development, biomass density or protective agent concentration (Hubalek, 2003; Morgan *et al.*, 2006; Prakash *et al.*, 2013).

The aim of this study was to evaluate the biomass production of four strains, *i.e.*, *Trichoderma atroviride* TRS14, *T. atroviride* TRS7, *Trichoderma simmonsii* TRS75 and *Trichoderma virens* TRS109, in the SSF cultures on lignocellulosic by-products used as media, and its subsequent preservation on these carriers in the process of lyophilization, in order to retain high survival and growth rates of strains in the obtained biopreparations.

Experimental

Materials and Methods

Microrganisms. Microbial material consisted of the following strains: *T. atroviride* TRS7, *T. atroviride* TRS14, *T. simmonsii* TRS75, and *T. virens* TRS109 (Culture Collection of Microbiology Lab, Research Institute of Horticulture, Skierniewice). Molecular identification of these strains, based on sequences of *ITS1* and *ITS2*, *tef1alpha*, *rpb2* and *chi18–5*, was described in the previous work (Skoneczny *et al.*, 2015; Oskiera *et al.*, 2015). The tested strains were stored on PDA (Potato Dextrose Agar) slants at 4°C.

Lignocellulosic by-products. The study was conducted with three lignocellulosic by-products (beet pulp, corn cobs and dried grass), in monocomponent and bicomponent cultures in the proportion of 1:1 of dried grass with beet pulp or dried grass with corn cobs.

Biomass production of *Trichoderma* fungi on lignocellulosic by-products in solid state fermentation (SSF). The agriculture by-products, initially dried and ground in a mill, were put into 1000 ml Roux flasks in the following amounts: cultures with a single component (beet pulp or corn cobs) – 80.0 g + 160 ml of distilled water, and (dried grass) – 40.0 g + 80 ml of distilled water; whereas in cultures with two components (dried grass with beet pulp and corn cobs, respectively) – 40.0 g + 80 ml of distilled water. The media were left for 60 minutes at room temperature, then moisture content was determined and sterilization was carried out at 121°C for 60 minutes. After sterilization, the media were incubated for 24 hours in the incubator at 25°C in order to control for sterility. The prepared media with mean moisture content in the range of 59.9–69.4% and a mean pH of 5.3–5.7 were inoculated with a standardized suspension of conidia in 0.1% Tween 80, obtained from 10-day fungal culture on PDA medium to the initial density of $1.3 \times 10^6 - 2.3 \times 10^6$ conidia/g dm of media and incubated for 10 days in the phytotron chamber at constant humidity of 75% and temperature 25°C. All cultures were performed in duplicate.

Preservation of the *Trichoderma* fungal biomass and spores on lignocellulosic carriers in the process of lyophilization. The lignocellulosic by-products overgrown with the tested strains (10.0 g) were introduced into 500 ml round bottom flasks together with 10 ml of 20% sterile solution of maltodextrin, used as the protective agent or an equivalent amount of sterile distilled water (control), and mixed thoroughly. The lyophilization process was preceded by pre-freezing to a temperature of –24°C, followed by freeze-drying for 20 hours at the external manifold of the Labconco Triad freeze dryer, at the pressure of 0.2 mbar. Once the process was complete, the lyophilizates were put into plastic bags and vacuum-sealed.

Evaluation of selected parameters of the growth kinetics of *Trichoderma* strains (in lyophilized biopreparations) using a Bioscreen C system. The parameters of the growth kinetics of *Trichoderma* strains in lyophilized biopreparations were analyzed using Bioscreen C system. Microcultures (five replicates each) were carried out in potato dextrose broth (PDB) under the following conditions: temperature of incubation 25°C, time of incubation – 3 days, continuous shaking, absorbance read at 540 nm, reading intervals every 20 minutes. Strains in SSF cultures prior to lyophilization were used as controls. Growth of the strains was recorded in the form of curves showing the dependence

of optical density against time. The selected parameters of growth kinetics (lag phase duration in h, the maximum rate of specific growth increase μ_{max} in h^{-1} and the maximum biomass yield ΔOD_{max} expressed as the difference between the maximum and minimum optical density) were determined based on the analysis of growth curves.

The length of the lag-phase was determined directly from the growth curves, whereas the other parameters – using the following formulas:

$$\mu_{max} = (lnOD_2 - lnOD_1)/(t_2 - t_1)$$

where:

OD_1 – optical density at the onset of the logarithmic phase

OD_2 – optical density at the end of the logarithmic phase

t_1 – onset of logarithmic phase

t_2 – end of logarithmic phase

$$\Delta OD_{max} = OD_{max} - OD_{min}$$

where:

OD_{max} – maximal value of optical density

OD_{min} – minimal value of optical density

Analytical methods. The number of viable cells, expressed as the number of CFU/g dm, in SSF cultures and lyophilizates (after rehydration in 10% maltodextrin solution or distilled water, respectively) was determined by the Koch plate method in potato dextrose agar medium (PDA) containing rose bengal (0.035 g/l) from three consecutive dilutions in 0.1% Tween 80 in triplicate.

The percentage of viable cells (% survivability) was expressed as a percentage of surviving cells compared to live cells prior to preservation.

Statistical calculations. Results pertaining to the number of CFU/g dm and growth rate of the tested strains in lyophilizates were statistically analyzed by three-way analysis of variance (type of medium, strain, biological product). The differences between means were determined by Duncan's test with a significance level of p = 0.05. The calculations were carried out using the Statistica 10 software (StatSoft).

Results

Biomass production of *Trichoderma* fungi on lignocellulosic by-products in solid state fermentation (SSF). In this study, four strains were used for the production of lyophilized biopreparations, including two strains of *T. atroviride* species (TRS14 and TRS7), one of *T. simmonsii* (TRS75) species and one of *T. virens* (TRS109) species, cultured on solid medium to obtain large amount of biomass. It was shown that the applied materials constituted suitable and inexpensive source of energy and nutrients for growing fungi. On monocomponent media (dried grass, beet pulp or corn cobs), the tested strains produced biomass in the amount of $8.6 \times 10^7 - 1.7 \times 10^8$ CFU/g dm. The exception was the strain *T. atroviride* TRS14, which grew poorly on corn cobs (7.4×10^6 CFU/g dm). For all the strains tested, the bicomponent substrates proved to be more efficient for biomass production, expressed as the number of CFU (either dried grass and beet pulp or dried grass with corn cobs). In this case, biomass production ranged from 1.7×10^8 to 5.3×10^8 CFU/g dm, depending on the strain. Strain *T. simmonsii* TRS75, was the most effective biomass producer on both, monoand bicomponent, substrates among the strains investigated ($1.1 \times 10^8 - 5.3 \times 10^8$ CFU/g dm). Three other strains, *i.e.*, *T. atroviride* TRS7, *T. atroviride* TRS17 and *T. virens* TRS109, produced comparable level of biomass ($9.3 \times 10^7 - 3.5 \times 10^8$ CFU/g dm) (Table I).

There was a slight increase in mean moisture content recorded after 10 days of SSF cultures within the range of 66.9–73.5% in comparison with mean initial values of this parameter (59.9–69.4%) (Fig. 1). A slight shift in the pH of medium was also observed after 10 days of culture – mean pH increased from the range 5.3–5.7 to 6.7–6.8 for mono- and bicomponent media, respectively (Fig. 2).

Preservation of the *Trichoderma* fungal biomass and spores on lignocellulosic carriers in the process of lyophilization. The biomass of tested strains obtained on lignocellulosic substrates was preserved by lyophilization, with the use of maltodextrin as the protective agent or with an equivalent amount of distilled

Table I
Biomass expressed as the number of cfu/g dm in 10-day SSF cultures of *Trichoderma* strains on lignocellulosic materials.

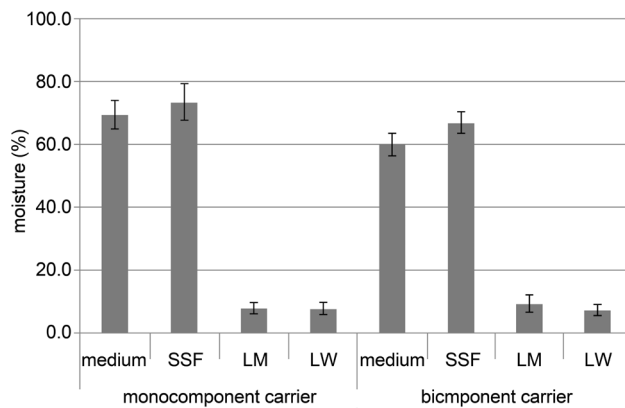| Strain	Monocomponent medium			Bicomponent medium	
	Dried grass	Beet pulp	Corn cobs	Dried grass + beet pulp	Dried grass + Corn cobs
T. atroviride TRS7	9.3×10^7	1.2×10^8	8.6×10^7	1.7×10^8	2.0×10^8
T. atroviride TRS14	1.1×10^8	1.3×10^8	7.4×10^6	2.2×10^8	3.5×10^8
T. simmonsii TRS75	1.7×10^8	1.1×10^8	1.2×10^8	5.3×10^8	2.8×10^8
T. virens TRS109	1.5×10^8	1.2×10^8	1.3×10^8	2.0×10^8	2.4×10^8

Fig. 1. Mean moisture content (%) of medium, after SSF cultures and of lyophilizates produced on the lignocellulosic materials with *Trichoderma* strains.

SSF – solid state fermentation (culture);
LM – lyophilizates with maltodextrin;
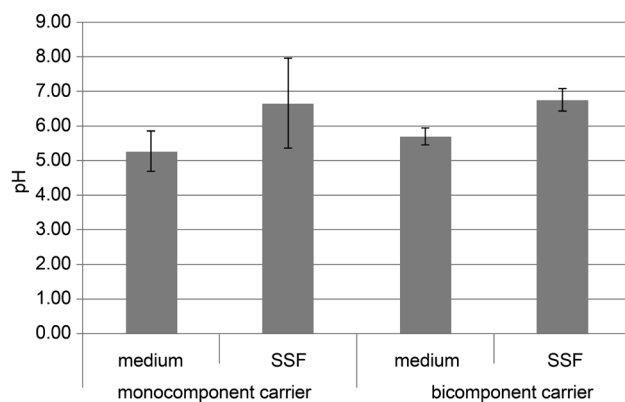LW – lyophilizates with distilled water (control)

Fig. 2. Mean pH value of medium and after SSF cultures on the lignocellulosic materials with *Trichoderma* strains.

SSF – solid state fermentation (culture)

water (control), by reducing the level of moisture of lyophilizates to a range of 7.9–9.4% (LM, lyophilizates with maltodextrin) and to range 7.3–7.8% for lyophilizates with water (LW, control) (Fig. 1). The lyophilized products demonstrated a significant reduction in the number of viable cells as compared to their amount prior to the preservation procedure. The biopreparations demonstrated survivability in the range from 40.89% to 48.43% of the CFU/g dm. The addition of a protective medium had no significant effect on the survivability of the tested strains. In lyophilizates with maltodextrin (0.84×10^8 CFU/g dm) or without it (0.62×10^8 CFU/g dm), similar count of viable cells was detected (Fig. 3). There were no significant differences in the survival of fungi in lyophilizates in relation to the test strain (54.99–70.27%) as well as the biomass carrier (56.88–75.45%). However, when considering the number of viable cells (CFU) in the preparations, but not their percentage compared with their initial number before lyophilization, a significant correlation was found between survival rate and both the test strain as well as the material used in the medium. A higher count of viable cells was observed in the biopreparation of the strain *T. simmonsii* TRS75 (1.48×10^8 CFU/g dm) as compared with preparations of other tested strains ($0.90 \times 10^8 – 0.97 \times 10^8$ CFU/g dm). Although the survival rate of strains on the monocomponent lignocellulosic carriers was higher (56.88–75.45%) than on the bicomponent compositions (58.57– 62.32%), the actual number of viable cells was higher on bicomponent carriers ($1.55 \times 10^8 – 1.68 \times 10^8$ CFU/g dm) compared with monocomponent ($0.59 \times 10^8 – 0.83 \times 10^8$ CFU/g dm), which was due to the higher initial CFU number of the tested strains in SSF cultures on mixed compositions
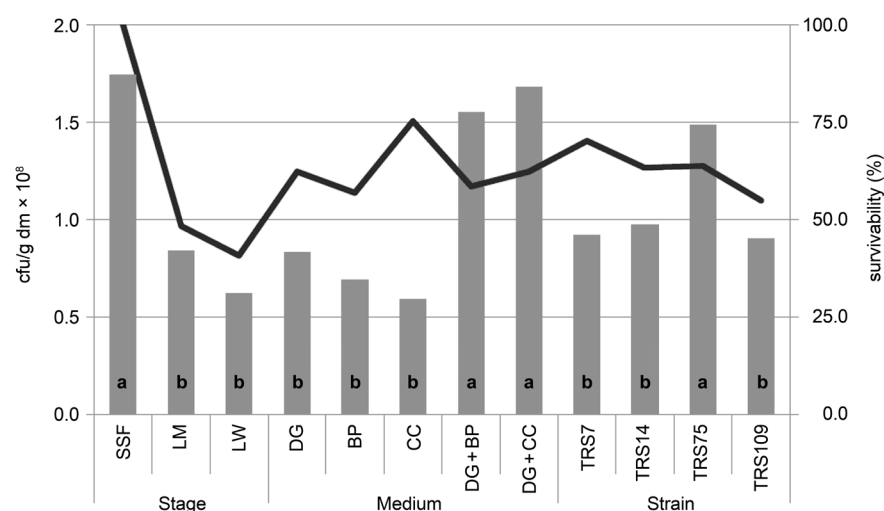

Fig. 3. The average values of cfu/g dm (bars) and survivability (line) of *Trichoderma* strains in SSF cultures and after lyophilization depending on the stage, medium and tested strain (three-way analysis of variance).

a, b – homogeneous groups according to the Duncan's test at p = 0.05

SSF – solid state fermentation (culture), LM – lyophilizates with maltodextrin, LW – lyophilizates with distilled water (control);
DG – dried grass, BP – beet pulp, CC – corn cobs

Table II
Mean values of selected parameters of the growth kinetics of *Trichoderma* strains depending
on the stage, medium and tested strain (three-way analysis of variance).

Variation factor		Dependent variables		
		Lag phase [h]	μ_{max} [h^{-1}]	ΔOD_{max}
Stage	SSF	15.16 a	0.1599 a	1.715 a
	LM	14.33 a	0.1528 a	1.548 a
	LW	14.56 a	0.1132 a	1.518 a
Medium	Dried grass	16.58 a b	0.2212 a	1.616 a
	Corn cobs	14.64 b c	0.1175 a	1.587 a
	Beet pulp	17.88 a	0.1277 a	1.497 a
	Dried grass + beet pulp	12.33 c	0.1156 a	1.675 a
	Dried grass + corn cobs	12.00 c	0.1279 a	1.594 a
Strain	*T. atroviride* TRS7	15.33 a	0.1179 a	1.573 a
	T. atroviride TRS14	13.31 a	0.1175 a	1.655 a
	T. simmonsii TRS75	13.66 a	0.1357 a	1.582 a
	T. virens TRS109	16.44 a	0.1969 a	1.566 a

a, b, c – homogeneous groups according to the Duncan's test at p = 0.05
SSF   – solid state fermentation (culture)
LM   – lyophilizates with maltodextrin
LW   – lyophilizates with distilled water (control)

of lignocellulosic by-products. In summary, the most effective monocomponent carrier that contributed to high survivability of the investigated strains (75.45%) was corn cobs, while the combination of this component with dried grass resulted in a slightly lower survival rate of the strains, *i.e.*, 62.32% (Fig. 3).

Evaluation of selected parameters of the growth kinetics of *Trichoderma* strains (in lyophilized biopreparations) using a Bioscreen C system. No significant influence was shown for the lyophilization process and the protective agent used in this procedure (maltodextrin) on the growth kinetics of selected parameters of the tested strains in the lyophilizates. The mean values of the lag phase length (14.33–14.56 h), the maximum specific growth rate (μ_{max} 0.1132 – 0.1528 h^{-1}) and the biomass yield expressed as ΔOD_{max} (1.518 – 1.548) for the strains lyophilized on lignocellulosic carriers, did not differ significantly from the values of the growth kinetics of strains in SSF control cultures prior to lyophilization (lag phase – 15.16 h, μ_{max} – 0.1599 h^{-1}, ΔOD_{max} – 1.715) (Table II).

Evaluating the growth kinetics parameters in relation to the composition of lignocellulosic by-products used as substrates during the production of biomass, and after lyophilization as its carriers as well as to the test strain, a significant effect of the preservation process was demonstrated only on the length of the lag phase, whereas there were no significant differences in the growth rates μ_{max} (0.1156 h^{-1} – 0.2212 h^{-1}) and biomass yield ΔOD_{max} (1.497 – 1.675). The shortest lag phases in cultures of tested strains were recorded in

lyophilizates on bicomponent media of corn cobs and beet pulp with dried grass (lag phase 12.00 – 12.33 h) and significantly longer in lyophilizates on beet pulp (17.88 h), dried grass (16.58 h) and corn cobs (14.64 h). Among the studied fungi, *T. virens* TRS109 had a longer lag phase (16.44 h) than the other strains analyzed (lag phase 13.31–15.33 h), but the differences between the strains were not statistically significant (Table II).

Discussion

Saprophytic fungi, mainly of *T. virens* species and *Trichoderma harzianum* species complex including *T. simmonsii*, have been successfully introduced as components of biopesticides in many countries, including Sweden (Binab T WG), Belgium (Trichodex, Biofungus), the USA (RootShield, PlantShield), or India (Bioderma Bioderma-H) (Kaewchai *et al.*, 2009; Chaverri *et al.*, 2015). In contrast, there are very few biocontrol products of domestic origin (*e.g.*, Vital Plus based on *Trichoderma viride*). Therefore, it is important to search for new native species, which could be used as a base for the production of biopreparations adapted to Polish conditions of agrarian cultivation. An important aspect of the production of such biopreparations, in addition to the selection of strains, is the use of appropriate methods of culture and inexpensive, readily available substrates, which would ensure a high biomass yield. Solid media are more effective in obtaining large amounts of microbiologically pure conidia (Lewis and

Papavizas, 1983), and their main advantage is the limited risk of contamination due to the low water activity.

This study used SSF culture technology applying lignocellulosic by-products, which constituted suitable culture environment for the production of biomass of filamentous fungi. The strains utilized biopolymers present in the media owing to numerous hydrolytic enzymes synthesized by them, including cellulases, xylanases polygalacturonases, laminarinasis and proteases that allow them to use hardly assimilable sources of carbon and nitrogen (Mitchell *et al.*, 2002). It was also found that more favorable for biomass production of *Trichoderma* strains were bicomponent compositions of dried grass with corn cobs and beet pulp $(1.7 \times 10^8 - 5.3 \times 10^8$ CFU/g dm) than the monocomponent media: beet pulp, corn cobs and dried grass $(7.4 \times 10^6 - 1.7 \times 10^8$ CFU/g dm), which was presumably associated with a more varied and diverse chemical composition of these substrates. This was more advantageous for obtaining higher biomass yield than in monocomponent substrates. Tewari and Bhanu (2004) cultured various species of *Trichoderma* fungi, including *T. harzianum*, on similar substrates as in the present study, *i.e.*, corn cobs and sugarcane, and obtained biomass yield of this species at 2.24×10^8 CFU/g dm and 2.79×10^8 CFU/g dm, respectively. Corresponding results were obtained in the previous study, where the *T. simmonsii* TRS75 strain cultured in media consisting of beet pulp or corn cobs, ensured biomass production at $1.1 \times 10^8 - 1.2 \times 10^8$ CFU/g dm. In the production of biopreparations, attention should be paid to the potential use of substrates with the addition of corn cobs in the SSF cultures of *Trichoderma* fungi, since it was found that the presence of this material, even as a sole carbon source in the medium, in addition to the production of biomass, had a positive effect on the synthesis of enzymes, including FP-ases, CMC-ases and xylanases (Kancelista and Witkowska, 2008).

When obtaining a high yield of the biomass, next to the proper composition of the substrate, it is important to determine the optimal parameters for the growth of filamentous fungi. In this work, the production of biomass of strains tested in SSF cultures lasted for 10 days at the temperature 25°C. Similar duration of the culture of filamentous fungi in SSF media (7 to 10 days) was determined by Kredics *et al.* (2003). The study of Orzua *et al.* (2009) demonstrated that the appropriate range of moisture (30–80%), dependent on the substrate, in the SSF cultures enhanced the efficient production of biomass. Moisture content of the substrates used in the present study ranged from 59.9% to 69.4%, which positively affected the growth of the studied fungi.

At high moisture content, lignocellulosic waste materials swell, thereby facilitating the access of filamentous fungi to polymers, which they are composed of. Fur-

thermore, the high water content improves the solubility of nutrients (Kovacs *et al.*, 2009; Xin and Geng, 2010).

Numerous authors, recommended the range of pH 4 to pH 6 as the optimum for the growth of filamentous fungi (Kredics *et al.*, 2003; Benitez *et al.*, 2004). In the current study, the initial pH value of the substrates was not adjusted, the mean pH of monocomponent media (dried grass, beet pulp or corn cobs) was 5.3, while the pH of bicomponent substrates (dried grass with beet pulp or dried grass with corn cobs) was 5.7. These pH values of substrates also facilitated the biosynthesis of extracellular hydrolytic enzymes, necessary for utilization of substrates, which resulted in a high biomass yield of the tested strains.

Majority of the publications have reported on the use of the following species as biological agents in the biocontrol products: *T. harzianum*, *T. virens*, *T. viride* and *Trichoderma koningi*, while *T. atroviride* was reported less commonly. The present study investigated two strains of the species: *T. atroviride*, TRS7 and TRS14, which similarly as strains of *T. simmonsii* TRS75 and *T. virens* TRS109, demonstrated a high biomass yield, particularly in the bicomponent lignocellulosic substrates $(1.7 \times 10^8 - 3.5 \times 10^8$ CFU/g dm). It has been demonstrated that these strains, by having a high capacity for breakdown of lignnocellulosic materials, also possess a potential as biological agents in biocontrol products for plant protection. Panahian *et al.* (2012) conducted a study on the formulation of biopreparations with *T. atroviride* and reached similar conclusions to ours.

One of the most important issues in studies on the production of biopreparations is their preservation intended for long-term storage. One of the preservation methods is lyophilization involving a variety of protective agents that is mainly applied to stabilize the biomass of pure cultures, including the industrially important strains of filamentous fungi, and allows for the long-term storage while maintaining their high survivability, good growth rate, genetic homogeneity and stability of technological traits. In the present work, the biomass of four strains tested, *i.e.*, *T. atroviride* TRS7, *T. atroviride* TRS14, *T. simmonsii* TRS75 and *T. virens* TRS109, obtained on lignocellulosic by-products, was preserved in the process of lyophilization with the addition of maltodextrin as a protective agent by reducing the moisture of the products to the desired range from 7.9% to 9.4%. Guijarro *et al.* (2006) reported that the moisture content of biopreparations produced on the basis of conidia stored at room temperature should be within the range of 5–15%.

While the survival rate of strains tested immediately after lyophilization depended solely on the preservation procedure of the biomass, there were no significant differences in the survivability of fungi with respect to the strains studied, the biomass carrier, or the

presence of protective medium. The study of Panahian *et al.* (2012) evaluated various protective agents including maltodextrin during the lyophilization process of species *T. atroviride* and *T. koningi* in SSF culture on molasses and corn grain, and also did not find a beneficial effect of maltodextrin on the survival of the controlled strains. In the current study, strains demonstrated survival rate in the range of 54.99% to 70.27% ($9.01 \times 10^7 - 1.48 \times 10^8$ CFU/g dm). The most effective biomass carrier in terms of the highest survival rate was corn cobs (75.45%), while beet pulp was the least efficient substrate (56.88%). Based on these results, it can be assumed that the applied lignocellulosic by-products were already relatively good preservation agents for retaining sufficient survivability of the strains tested as well as the high growth rate.

After lyophilization, the tested strains exhibited proper growth rates. The only significant differences were recorded in the length of the lag phase, depending on the biomass carrier. Longer adaptive phase had no effect on the maximum rate of specific growth ($\mu_{max} = 0.1156\ h^{-1} - 0.2212\ h^{-1}$) and the final maximum biomass yield ($\Delta OD_{max} = 1.497 - 1.675$) of the strains analyzed. The results obtained are a continuation of previous studies. In the previous studies (Kancelista *et al.*, 2013) we showed an increased growth rate and higher biomass yield of *T. harzianum* and *T. virens* strains that grew on lignocellulosic substrates ($\mu_{max} = 0.135\ h^{-1} - 0.194\ h^{-1}$ and $\Delta OD_{max} = 1.660 - 1.786$) when compared to strains cultured on a standard fungi medium (PDB $\mu_{max} = 0.081\ h^{-1} - 0.136\ h^{-1}$ and $\Delta OD_{max} = 1.397 - 1.479$). However, after the preservation of the biomass in the drying process, these strains were characterized by the reduced values of growth kinetics parameters. Simões *et al.* (2009) controlled the influence of different carbon sources on the growth of *T. viride* strain during the 60-hour culture in a Bioscreen C system. The lowest biomass yield was reported in cultures with glucose $\Delta OD_{max} = 1.100$, while the highest in the culture with sorbitol $\Delta OD_{max} = 2.100$. Other authors (Rossi-Rodrigues *et al.*, 2009) evaluated the growth of *Trichoderma* fungi of species *Trichoderma hamatum*, *T. harzianum*, *T. viride* and *Trichoderma longibrachiatum*, in relation to the carbon sources (glucose, sucrose) as well as nitrogen sources (yeast extract and tryptophan). They found that the growth rate was a species-specific trait, and the most effective carbon source was glucose in combination with yeast extract, on which the *T. hamatum* strain ensured biomass yield at the approximate level of $\Delta OD_{max} = 1.800$, and the *T. harzianum* strain at $\Delta OD_{max} = 1.500$. In the present study, the strain of *T. simmonsii* species derived from lyophilizates obtained on lignocellulosic substrates showed a slightly higher biomass yield ($\Delta OD_{max} = 1.582$) than in the above-discussed study. The remaining test strains grew to the ΔOD_{max} level of 1.556–1.655.

These results demonstrated the preservation of the high growth rate after the process of lyophilization of the investigated strains. In order to find a commercial application in plant protection for biopreparations based on *Trichoderma* strains, it is necessary to provide a high survival rate and the stability of all the important parameters for at least 18 months of storage (Pedreschi and Aguilera, 1997). Therefore, further studies will focus on the control of viability and retaining the ability for the biosynthesis of enzymes (mainly related to the degradation of cell wall of phytopathogens) during long-term storage of fungal preparations at room temperature and comparatively at refrigerated conditions.

Summary

In conclusion, it was shown that the applied lignocellulosic by-products such as dried grass, beet pulp and to a lesser extent, corn cobs were suitable substrates for the production of *Trichoderma* biomass. However, bicomponent media, composed of dried grass and beet pulp and dried grass with corn cobs, were more effective for fungal growth, compared to monocomponent media. The process of lyophilization of the resultant biomass reduced the survival rate of the strains present in lyophilized biological products by about 50–60%. However, the actual number of viable cells in lyophilized biopreparations was relatively high ($0.58 \times 10^8 - 1.68 \times 10^8$ CFU/g dm). Maltodextrin used in the lyophilization process had no particular protective effect on the survival rate of the strains. A very similar number of viable cells was detected either in the presence or absence of maltodextrin in the lyophilizates on lignocellulosic carriers ($0.62 \times 10^8 - 0.84 \times 10^8$ CFU/g dm).

This paper presents the results not yet described in the literature and concerning the use of lignocellulosic by-products as carriers of spores and fungal biomass of *Trichoderma* both in the process of their production and preservation by freeze-drying. Studies on the stability of such biopreparations are continued, as one of the most important issues is to maintain the high survivability and growth rate of the fungi in biological products during their long-term storage.

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