AGRICULTURAL SCIENCE AND TECHNOLOGY, VOL. 14, No 2, pp 75-80, 2022
Published by Faculty of Agriculture, Trakia University, Bulgaria
ISSN 1313-8820 (print)
ISSN 1314-412X (online)
http://www.agriscitech.eu
DOI: 10.15547/ast.2022.02.022

Monitoring the productivity of *Trichoderma viride* strain in submerged cultivation

O. Georgieva*, N. Karadzhova

Maritsa Vegetable Crops Research Institute (MVCRI), 32 Brezovsko shosse, 4003 Plovdiv, Bulgaria

(Manuscript received 19 July 2021; accepted for publication 14 April 2022)

Abstract. *The influence of the nutrient medium, the amount of inoculate and the initial acidity on the accumulation of the biomass of the antagonistic fungus Trihoderma viride during submerged cultivation was studied. As a result of the conducted research, biotechnological parameters were optimized for the production of a biological product by the method of submerged cultivation. A balanced semi-synthetic nutrient medium has been developed in which the fungus performs a full cycle of ontogenesis, forming chlamydospores and phialoconidia. The produced biomass with a titer of more than 1.10^6 chlamydospores in 1 ml can be used both for the production of liquid form of the preparation and for the production of preparations on solid substrates.*

Keywords: *Trihoderma viride*, submerged fermentation, parameters, medium composition, accumulation of biomass

Introduction

Despite numerous studies related to the use of various strains of microorganisms as producers of biological products, in practice, only a few antagonistic strains of the genera *Bacillus*, *Penicillium*, *Pseudomonas*, *Streptomyces*, *Chaetonfium* and *Trichoderma* have commercial value (Elad et al., 1981; Ait-Lahsen et al., 2001; Litovka, 2002; Kolombet, 2004; Cornejo et al., 2009; Ziganshin et al., 2020). The most advanced technologies are those to produce enzymes based on different strains of *Trichoderma* spp. with the final product - a metabolite synthesized by the producer strain. The main purpose of these technologies is to obtain enzymes (or antibiotics), and the culture of the fungus serves as a "bioreactor" synthesizing the desired product (Tangnu et al., 2004; Zhou et al., 2008). At the end of the cultivation process, the biomass of the producer strain is subject to disposal (Castanon and Wilke, 2004; Zayed, 2005).

When developing a technology to produce products based on the biomass of fungi (of the genus *Trichoderma*) a primarily different task is set at the lowest price of raw materials and energy resources to obtain the maximum possible amount of biologically active biomass capable of long-term storage (Gomes et al., 2008; Schneider**,** 2011).

Technologies to produce preparations based on filamentous fungi can be divided into two different types of cultivation: surface (solid phase) and submerged - in a liquid nutrient medium. Technologies are also available that combine these two methods (Gromovykh et al., 2002). In the case of traditional solid-phase technology of production on solid nutrient medium, the final product contains a relatively small amount of fungal conidia and their metabolites, and a large volume of quantitative substrates used for fermentation.

The cultivation of mycelial fungi by the submerged method is considered a more technological process (Waghunde et al., 2016; Shternshis**,** 2012). The controlled conditions of the fermentation process allow a standardized final product to be obtained (Mendelsohn and Dinar, 2003). In addition, cultivation in a closed apparatus excludes the penetration of air into the working area of the producer strain, which is why it can be considered more environmentally friendly.

Our study was aimed at monitoring the productivity of the *Trichoderma viride* strain (biomass accumulation) on different media by the method of submerged cultivation.

Material and methods

Trichoderma viride cultivation scheme.

- Strain growth on sloping potato-dextrose agar.
- Preparation of starter material for submerged cultivation.
- The main production of the strain on a shaker.

*e-mail: olgaizk@abv.bg

Strain-producer

The strain *Trichoderma viride* No12 from the collection of fungal antagonists of Maritsa Vegetable Crops Research Institute (MVCRI) was used for the research.

Inoculation and fermentation nutrient media for cultivation of the studied producer strain

Several nutrient media were tested to determine the medium for optimal growth of the *Trihoderma viride*. Composition of nutrient media (g /L) of sterilized water:

Water agar (A) – agar-agar, 20 g; Oat agar (OA)- liquid decoction of oat, agar-agar, 20 g; Potato agar (PA) - liquid decoction of potato, agar-agar, 20 g; Potato-glucose agar (PGA) - liquid decoction of potato, glucose 20 g, agar-agar, 20 g; Potato-dextrose agar (PDA)- liquid decoction of potato, dextrose, 20 g, agar-agar, 20 g; Corn agar (CA) - liquid decoction of corn, agar-agar, 20 g; Czapek Doxe agar (CzA)- sucrose, 30.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; $FeSO_47H_2O$, 0.01; KCL, 0.5; agar-agar, 20.0 g.

Average linear growth (ALG) was calculated by using the formula: (ALG) = (C_{10} – C_1)/ 9, where C_{10} = colony diameter after 10 days, C_1 - colony diameter after one day of incubation.

• Culture medium: PDA (standard).

• Fermentation media: Potato-dextrose broth (PDB) - prototype, standard, Czapek cultural broth – (CzB) synthetic nutrient medium, Wheat bran broth (WB)- semi-synthetic nutrient medium.

Composition of nutrient media, g/L of sterilized water: (PDB) - Potato extract - liquid decoction of potato, dextrose, 20 g. (CzB): sucrose, 30.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; $FeSO_47H_2O$, 0.01; KCL, 0.5. (WB) - wheat bran – 50g, $NaNO_3$ - 9g.

Starter material

• Pure culture of *Trihoderma viride* was grown on potato-glucose agar for 7 days at + 28°C to form abundant conidial cover.

• Preparation of vegetative cells material - on a shaker Appetitions-Schüttelschrank BS-4 B. Braun (120 rpm) at a temperature of +28°C for 48, 72, 96 and 120 hours. For this purpose, flasks with 100 ml of cultutal broth (prototype, synthetic, semi-synthetic) were used, on which a culture of pure *Trihoderma viride* culture was made.

Cultivation of the producer strain (main production) was carried out on a Schüttelschrank BS-4 B. Braun Incubations- shaker (120 rpm) at 28°C for 96 hours. The titer of phylaconidia and chlamydospores of the fungus *Trihoderma viride* was determined after the end of fermentation in the Fuchs-Rosenthal chamber according to the formula: X = a x 400 / b x 10^3, CFU / ml, where X is the estimated number of spores; a - the number of spores in a certain volume of the chamber; b - the number of squares. The experiments were repeated 3 times.

Microscopic control of the fermentation process: microscopic objects were prepared to monitor the development of the strain.

Influence of active initial acidity on biomass accumulation. The experiment had eight options - from pH 3.5 to pH 7.0 with a pH range of 0.5.

Determination of the amount of biomass in the culture broth (mg/ml) during cultivation

The culture fluid was centrifuged after 48, 72, 96 and 120 hours.

Influence of the amount of starter material on the accumulation of biomass

The spore suspension of the strain *Trihoderma viride* with a titre of 1.10^6 conidia in millilitre (CFU/ml) was inoculated on liquid Potato-glucose nutrient medium (PGM), the amount of suspension varying from 1% to 10% by volume of the culture medium. The experiments were repeated 3 times.

Statistical processing of the results of all data was carried out according to standard accepted methods (Dospechov, 1985). The average values and the mean square deviation were calculated to determine the confidence interval at the 95% significance level. Statistical processing (variation and analysis of variation in the results) was carried out using the computer program SPSS Statistics version 17.0 and Excel 2007.

Results and discussion

Storage and production of a strain of Trihoderma viride. Influence of nutrient media, cultivation temperature and lightening mode on mycelia growth and spore formation

There are many works devoted to the study of conditions favorable for the active growth of mycelium and spores of various species of fungi, the results of which are summarized in a number of monographs (Bilay, 1982; Bilay, 2000; Chang and Miles, 2004; Stamets, 2000). They indicate a sufficiently large range of fluctuations in the optimal values of temperature and pH of the medium for the growth of different strains of the same species, which requires appropriate studies for each potential producer.

Our data are quite consistent with the information given in the literature (Kolombet, 2004) regarding commercial conditions favorable for the growth of mycelium of *Trichoderma* species. As a result of testing several nutrient media, the optimal one for maintaining the *Trihoderma viride* strain was determined (Figure 1). The maximum growth of the *Trichoderma viride* mycelium with a growth rate above 8 mm/24 h is observed on the 9th day after sowing the fungus on Czapek Dox agar and potato glucose agar. The growth rate of the *Trichoderma viride* strain on oat and corn agar is 6-7 mm/24 hours. The results obtained suggest that such affordable and inexpensive media as (PA), (OA), (PDA) and (CzA) can be used in the production of the starting mycelium *Trichoderma viride*.

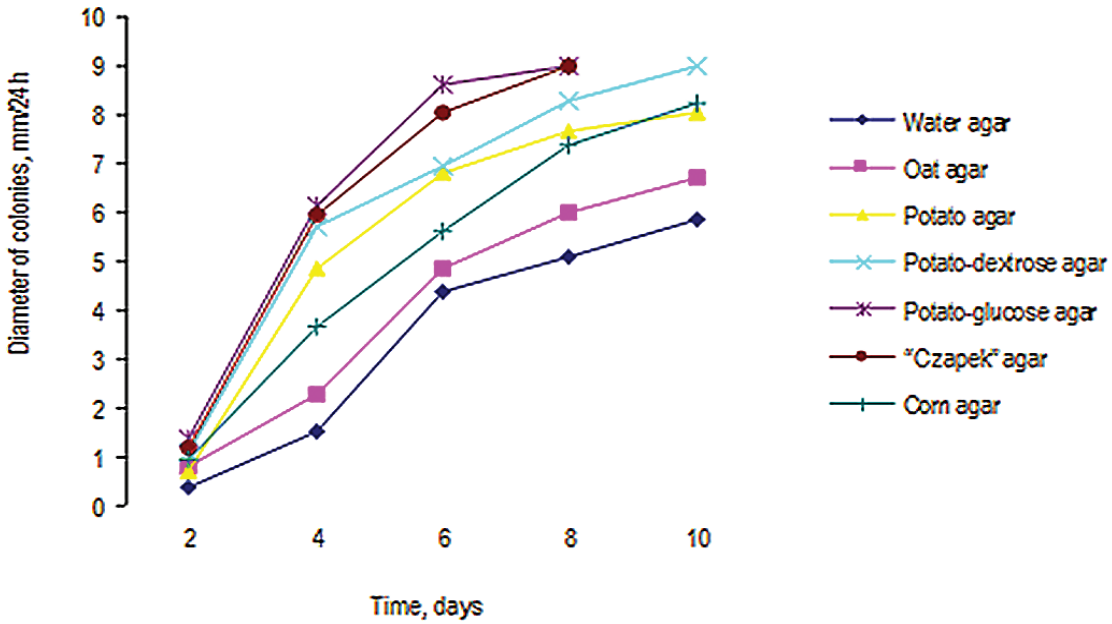

Figure 1. Influence of nutrient media on mycelia growth of *Trihoderma viride.*

As a result of our research, it was found that physical factors such as temperature, pH and light affect the growth rate of vegetative mycelium of fungi and their ability to sporulate. It was found that on a standard potato glucose agar, the maximum growth of the *Trihoderma viride* mycelium is observed in the temperature range 27°C-29°C (11.1 mm/ 24 h), active spore formation (12,5x10^3 CFU/ml) - at a temperature of 27°C (Figures 2 and 3).

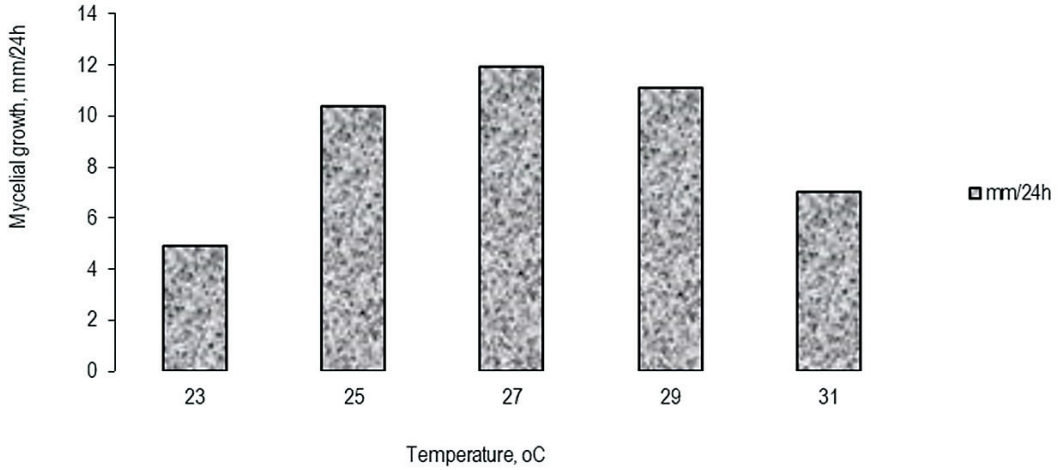

Figure 2. Influence of temperature on mycelia growth of *Trichoderma viride.*

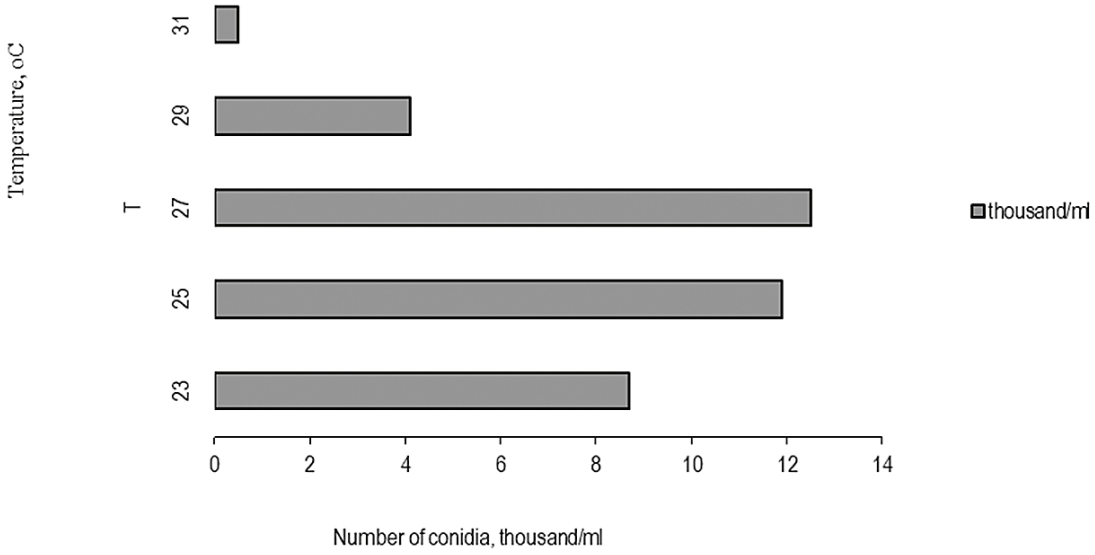

Figure 3. Influence of temperature on spore formation in *Trihoderma viride*.

The lighting mode is an important factor affecting the growth of the *Trihoderma viride.*The best growth of the mycelium of *Trihoderma viride* is manifested in conditions of constant darkness - 11.9 mm/24 h (Table 1), and the weakest growth of the mycelium - in conditions of constant light - 7.9-8.6 mm/24 h. The growth rate of the mycelium in this light mode is close to that in conditions of constant darkness - 11.2-11.4 mm/24h. The effect of light on the intensity of spore formation in *Trihoderma viride* is similar in the modes of constant light, constant darkness and variable light - night for 12 hours.

Table 1. Mycelium growth and the number of *Trichoderma viride* conidia in different lighting conditions (Temperature + 24°C)

Lighting mode	Constant light	Constant darkness	12 h light + 12 h darkness
Mycelial growth, mm/24h	7.9 ±0.3	11.9± 0.1	11.4± 0.1
Number of conidia, CFU/24h	3146±0.6	10984±0.3	8022±0.1

Production of starter material

Influence of nutrient media on biomass formation of Trihoderma viride

According to the literature data, Trichoderma strains produce conidiospores massively on solid media and form many chlamydospores when deep cultured in liquid media. The dynamics of spore accumulation depends on the sources of nutrients - organic nitrogen, carbon, ammonium nitrate, and ammonium sulfate (Kusakabe et al., 1979; Rodriguez and Pioneros, 2007; Kocher et al., 2008). Nitrogen has great influence on the formation of conidia in mycelial fungi. For maximum accumulation of biomass, the optimal ratio of carbon to nitrogen (C: N) in the nutrient medium is of great importance (Elad et al., 1981; Grinko, 2004).

As carbon source, we used contrasting fermentation media: potato-glucose (Prototype), synthetic Czapek medium (SNM), and semi-synthetic nutrient medium (SSNM) with the nitrate form of nitrogen $NaNO_3$ and wheat bran. As a result of the study, it was found that most of the biomass is formed when cultivated on potato-glucose and semi-synthetic medium with wheat bran - 20 g/L and 28 g/L after 96 h and 31 g/L and 28 g/L after 120 h of cultivation, respectively (Table 2).

Table 3 presents the results of a study of the effect of nutrient media on the accumulation of phialoconidia and chlamydospores of *Trihoderma viride*.

Table 2. Accumulation of *Trichoderma viride* biomass on various types of media

Nutrient media	*Exposition, (h)	** Amount of biomass, g/ L
Prototype	72	20.20±0.3
Prototype	96	25.70±1.4
Prototype	120	31.30±1.4
Synthetic nutrient medium	72	5.40±0.1
Synthetic nutrient medium	96	12.19±0.6
Synthetic nutrient medium	120	14.37±0.3
Semi-synthetic nutrient medium	72	22.16±7.0
Semi-synthetic nutrient medium	96	28.00±1.4
Semi-synthetic nutrient medium	120	28.00±1.4

Values are means of triplicate ± standard deviation.
Note: Prototype, standard (Potato-glucose broth), Synthetic nutrient medium (Czapek cultural broth), Semi-synthetic nutrient medium (Wheat bran broth).

Table 3. Accumulation of *Trihoderma viride* spores on various nutrient media

Productivity	Type of media		
	(Prototype)	(Synthetic nutrient medium)	(Semi-synthetic nutrient medium)
Titer of conidiospores, $1x10^6$ CFU/ml	70.0±1.75	42.5±1.63	4.8±0.96
Titer of chlamydospores, $1x10^6$ CFU/ml	2.0±0.45	4.8±1.22	29.5±1.29

Note:values are means of triplicate ± standard deviation

The results of the experiment showed that the concentration of phialoconidia in Prototype nutrient medium is 1.5 higher than in Semi-synthetic nutrient medium. The amount of chlamydospores is less in all variants except the Prototype variant. Thus, for the cultivation of *T. viride*, a balanced semi-synthetic nutrient medium on which the fungus carries out a complete cycle of ontogenesis, forming chlamydospores and phialoconidia. Since these structures ensure the survival of fungi under unfavorable conditions, biological products are usually created on their basis, which allows providing effective storage of the biopreparation prior to use.

Influence of the amount of starter culture on the accumulation of biomass

The accumulation of biomass does not significantly depend on the amount of starter material added to the medium (Kocher et al., 2008). We have established that the optimal amount of seed material for intermediate cultivation was 2.5%. With this amount of primary inoculum, 96 mg/ml of biomass accumulates after the completion of the full cultivation cycle. These indicators do not differ significantly from those in the variants with 5% and 10% starter material content. In all variants, the formation of conidia spores is observed, and in the 4th the presence of chlamydospores is observed (Table 4).

Table 4. Influence of the percentage of spore suspension on the accumulation of biomass and the number of spores during intermediate cultivation

Spore suspension, %	Absolutely dry biomass, mg/ml	Titer, CFU /ml	
		Conidiospores	Chlamydospores
1.0	46±0.07	1.1 x 10^3	0.0
2.5	96±0.01	9.3 x 10^3	0.0
5.0	97±0.03	2.8 x 10^4	0.0
10.0	99±0.05	4.1 x 10^4	+

Effect of active initial acidity on biomass accumulation

Judging by the literature data, the optimal pH value of nutrient media intended for the cultivation of filamentous fungi lies in the pH range of 5-6 (Juhasz et al., 2004; Ziganshin et al., 2020). We studied the effect of the initial pH of the medium on the accumulation of biomass from pH 3.5 to pH 7.0 with a pH range of 0.5. As a result, it was found that the optimal pH values for the maximum accumulation of *Trihoderma viride* biomass are about 5.5. If the pH value deviates from this value, the amount of biomass decreases. Under the microscope, conidia are mainly formed in the pH range of 5.5 - 6.5. This confirms that the most suitable initial acidity is in the pH range of 5.5 - 6.5 (Table 5).

Table 5. Dynamics of *Trichoderma viride* biomass accumulation

Culturing time, t/h	pH		Biomass, g/L	
	Prototype*	SSNM**	Prototype*	SSNM**
24	6.14±0.19	6.96±0.07	20.20±0.3	28.0±1.4

Note: Values are means of triplicate ± standard deviation
t/h - time of cultivation, * Prototype - Potato-glucose nutrient medium, ** Semi-synthetic nutrient medium - Wheat bran medium, 95% confidence interval

Conclusion

A balanced semi-synthetic nutrient medium in which the *Trichoderma viride* performs a full cycle of ontogenesis, forming chlamydospores and phialoconidia has been developed. The medium is optimized for the main technological parameters of cultivation (food sources, amount of inoculate, initial acidity, cultivation time), it provides the biomass of the *Trichoderma viride* in the form of mycelium and chlamydospores or phialoconidia. Optimum pH and temperature for biomass and spore production by *Trichoderma viride* was 5.5 and 28°C. Under optimized culture conditions for 96 h, the accumulation of biomass in the form of mycelium is 28 g/L, phialoconidia with a titer of 4.8x10^6 CFU/ml and chlamydospores with a titer of 29.5x10^6 CFU/ml.

Acknowledgements

This study is part of the National Science Program „Healthy Foods for a Strong Bio-Economy and Quality of Life“ (NNP-FOOD).

References

Ait-Lahsen H, Soler A, Rey M, De La Cruz J, Monte E and Liobell A, 2001. An antifungal glucanase from the biocontrol fungus *Trichoderma harzianum*. Applied and Environmental Microbiology, 67, 5833-5869.

Bilay V, 1982. Methods of experimental mycology. A Reference book, Naukova dumka, Kiev, 583 p.

Bilay V, 2000. Growth of edible mushrooms on commercial agar media. Mushroom Sci. Ed. L. Van Griensven. Maastricht (Netherlands), Balkena, 15, 779-782.

Castanon M and Wilke CR, 2004. Effects of the surfactant Tween 80 on enzymatic hydrolysis of newspaper. Bioengineering and Biotechnology, 23, 1365-1372.

Chang ST and Miles PhG, 2004. Mushrooms. Cultivation, nutritional value, medicinal effect and environmental impact. London, CRC Press, 451 p.

Cornejo P, Rubio R and Borie F, 2009. Mycorrhizal propagule persistence in a succession of cereals in a disturbed and undisturbed andisol fertilized with two nitrogen sources. Chilean Journal of Agricultural Research, 69, 426-434.

Dospechov B, 1985. Methods of field experience. Moscow, Agropromizdat, 351 p. (Ru).

Elad Y, Chet I and Henis Y, 1981. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica, 9, 59-67.

Gomes I, Shaheen M, Rahman SR and Gomes DJ, 2008. Comparative studies on production of cell wall-degrading hydrolases by *Trichoderma reesei* and *T. viride* in submerged and solid-state cultivations. Bangladesh Journal of Microbiology, 23, 149-155.

Grinko N, 2004. Biotechnological aspects of cultivation of the *Thrichoderma harzianum* Rifai strain VKM F-2477D. Bulletin of the Russian Academy of Agricultural Sciences, 1, 57-61 (Ru).

Gromovykh T, Malinovsky A and Koryanova T, 2002. Effect of different forms of fungal biopreparation for increasing quality of *Picea obovata* seedlings. In: Dumroese, R. K.; Riley, L. E.; Landis, T. D., technical coordinators. National proceedings: forest and conservation nursery associations-1999, 2000, and 2001. Proceedings RMRS-P-24. Ogden, UT: U.S. Department of Agriculture Forest Service, Rocky Mountain Research Station, p. 231.

Juhasz T, Szengyel Z, Szijart N and Réczey K, 2004. Effect of pH on cellulase production of *Trichoderma ressei* RUT C30. Applied Biochemistry and Biotechnology, 113, 201-211.

Kocher G, Kalra K and Banta G, 2008. Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. Internet Journal of Microbiology, 5.

Kolombet LV, 2004. Preparation on the basis of *Trichoderma asperellum* in the system of biological protection of wheat from Fusarium ear scab. Journal of Agriculture and Natural Sciences of Zhejiang University. Proceedings of the Eighth International Seminar on *Trichoderma* and *Gliocladium*, 30, 394-395.

Kusakabe C, Kodama K, Kununaka A, Yoshino H and Soda K, 1979. Extracellular producion of L-lysine-□-oxidase in wheat bran culture of a strain of *Trichoderma viride*. Agricultural and Biological Chemistry, 43, 2531-2533.

Litovka Y, 2002. Influence of biocontrol strains *Trichoderma asperellum, Bacillus subtilis* and *Pseudomonas fluorescens* on biological activity and structure of soil biota. In: Siberian Journal of Ecology, 3, 371-376 (Ru).

Mendelsohn R and Dinar A, 2003. Climate, water, and agriculture. Land Economics, 79, 328-341.

Rodriguez I and Pioneros Y, 2007. Production of enzymatic complex in solid state fermentation by *Trichoderma* sp. using palm oil empty fruit bunch (EFB) as substrate. Vitae [online], 14, 35-42. ISSN 0121-4004.

Schneider Y, 2011. Metabolic stability of soil saprophyte Trichoderma spp. in association with other soil fungi. In: Abstract's Book. International conference: Ecology of soil microorganisms, Prague, Czech Republic, pp.131 (Ru).

Shternshis M, 2012. Trends of microbial pesticides biotechnology developed for plant protection in Russia. Tomsk State University, Journal of Biology, 2, 92-100 (Ru).

Stamets P, 2000. Growing gourmet and medicinal mushrooms. Ten Speed Press, CA, 554 p.

Tangnu SK, Blanch HW and Wilke CR, 2004. Enhanced production of cellulase, hemicellulase, and β-glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnology & Bioengineering, 23, 1837-1849.

Zayed G, 2005. Bio-active composts from rice straw enriched with rock phosphate and their effect on the phosphorous nutrition and microbial community in rhizosphere of cowpea. Bioresource Technology, 96, 929-935.

Zhou J, Wang YH, Chu J, Zhuang YP, Zhang SL and Yin P, 2008. Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14. Bioresource Technology, 99, 6826-6833.

Ziganshin DD, Egorshina AA, Luk'yantsev MM and Sirotkin AS, 2020. Pilot submerged cultivation of *Trichoderma asperellum* RNCIM F-1323 micromycete. Proceedings of Universities. Applied Chemistry and Biotechnology, 10, 39-47.

Waghunde RR, Shelake RM and Sabalpara AN, 2016. Trichoderma: A significant fungus for agriculture and environment. African Journal of Agricultural Research, 11, 1952-1965.