ISSN 1313 - 8820 (print)
ISSN 1314 - 412X (online)
Volume 9, Number 3
September 2017

AGRICULTURAL SCIENCE AND TECHNOLOGY

2017

An International Journal Published by Faculty of Agriculture,
Trakia University, Stara Zagora, Bulgaria

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The articles appearing in this journal are indexed and abstracted in: DOI, EBSCO Publishing Inc., AGRIS (FAO) and DOAJ.

The journal is accepted to be indexed with the support of a project № BG051PO001-3.3.05-0001 "Science and business" financed by Operational Programme "Human Resources Development" of EU. The title has been suggested to be included in SCOPUS (Elsevier) and Electronic Journals Submission Form (Thomson Reuters).

This issue is printed with the financial support by Contract No DNP 05-21/20.12.2016, financed from Fund 'Scientific Research' grant Bulgarian scientific Periodicals.

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ISSN 1313 - 8820 (print)
ISSN 1314 - 412X (online)

Volume 9, Number 3
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AGRICULTURAL SCIENCE AND TECHNOLOGY, VOL. 9, No 3, pp 213 - 217, 2017
DOI: 10.15547/ast.2017.03.039

Influence of organic nitrogen amendment, containing amino acids on the cellulase and xylanase, produced by *Trichoderma* spp. isolates

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(Manuscript received 14 June 2017; accepted for publication 8 August 2017)

Abstract. *Cellulases and hemicellulases are amount the main hydrolytic enzymes, involved in the bioconversion of lignocellulose material by microorganisms. Filamentous fungi of the genus Trichoderma are one of the most studied and good producer of cellulases and hemicellulases. The nutrients balance, especially carbon to nitrogen ratio, is one of the main factors of the biodegradation. The ability of 37 local isolates of Trichoderma sp. to produce cellulases and xylanase were tested in solid state cultivation on wheat straw as a substrate whit two variants: 1. the straw was only moistured with destilated water (CN 80:1); 2. the C:N ratio of the straw was adjusted to 30:1 using organic nitrogen amendment. There is a significant difference in the enzymatic activity of the isolates in their cultivation on straw with CN 80 and CN 30. The highest carboxymethylcellulase (CMCase) activity at CN 80 showed T1T (110.19U/ml), and in the variant at CN 30 - TD (369.07U/ml). The highest β-glucosidase activity on both variants CN 80 and CN 30 was established for TG (2743.1U/ml - 12679.9U/ml). The highest xylanase activity at CN 80 and CN 30 was measured on T4I (21311.5U/ml – 47937.5U/ml). After ONA addition, all enzymes activities have increased several times, indicating the enhancing effect of the additive. The average activity of CMCase increased 6.1 times, the average β - glucosidase activity increased 5.1 times, while the xylanase activity increased 4.9 times for all tested isolates. The increase in activity of the investigated enzymes showed different patterns.*

Keywords: *Trichoderma,* endoglucanase, xylanase**,** β-glucosidase, bioconversion of lignocellulose

Abbreviations: β-Glu - β-glucosidase; DNSA - dinitrosalicylic acid; CMC - carboxymethyl cellulose; CMCase - Carboxymethyl cellulose; CN ratio - carbon to nitrogen ratio; MUB - modified universal *buffer*; ONA - Organic nitrogen amendment; PNG - p-Nitrophenyl-β-D-glucoside; PNP - p-Nitrophenol; Xyl – xylanase

Introduction

Lignocellulose is the major component of biomass produced by photosynthesis and it is the most abundant agricultural residue in the world. Lignocellulose material is composed of three types of polymers – cellulose, hemicellulose and lignin, which are chemically bonded and strongly intermeshed. Cellulose and hemicellulose are polymer macromolecules which consist of different sugars; whereas lignin is an aromatic, phenylpropanoid polymer synthesized from the phenolic precursors of coniferyl, synapyl and p-coumaryl alcohol. The content of cellulose, hemicellulose and lignin in plant materials varies within the following limits: cellulose - 25 ÷ 55%; hemicellulose - 25 ÷ 50%; lignin - 10 ÷ 35% (Betts et al., 1991). Cellulose is the most abundant biopolymer on Earth. It is a linear polymer of D-glucose units linked by β- 1.4-glycosidic bonds and is crystalline in nature. Hemicellulose is the second most common polysaccharide in nature. It consists of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by β-1.4- and occasionally β -1.3-glycosidic bonds. Xylan is the main carbohydrate found in hemicellulose. Xylan has a complex structure consisting of β-1.4-linked xylose residues in the backbone (Coughlan and Hazlewood, 1993).

Fungi are the best-known microorganisms capable of degrading these three polymers. Microorganisms have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases and it is responsible for cellulose and hemicellulose degradation; and a unique oxidative and extracellular ligninolytic system, that depolymerizes lignin.

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Hydrolysis of xylan and cellulose is the main step for the efficient utilization of lignocellulosic materials in nature. Filamentous fungi were reported as good producers of lignocellulolytic enzymes because of the extracellular release of the enzymes, higher yield compared to bacteria and yeast, and also the production of several auxiliary enzymes that are necessary for better degradation of polysaccharides (Ja'afaru, 2013).

Cellulase is a complex enzyme composed of exocellobiohydrolase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21), which act synergistically in the process of conversion of the cellulose to smaller sugar components. Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa (Lynd et al., 2002; Peciulyte, 2007).

Endoxylanases (β-1,4-D-xylan xylanoxylohydrolase; EC 3.2.1.8) are the main enzymes involved in xylan hydrolysis causing a decrease degree of polymerization of the substrate and liberating shorter oligomers, xylobiose and even xylose. β-1.4–xylosidase (β-1.4-D-xyloside xylohydrolase; EC 3.2.1.37) that hydrolyses the small oligosaccharides and xylobiose to xylose is an important enzyme when complete hydrolysis of xylan is needed (Nour El-Dein et al., 2014).

Xylanases and cellulases has been considered as the most important enzymes for the bioconversion of lignocellulosic materials, especially agricultural residues and wastes to produce higher value products such as enzymes for industrial application, ethanol fuel and other chemicals (Khokhar et al., 2012).

Significant amount of unused straw biomass is produced as

waste products from agricultural production annually. Incorporation of straw into the soil is an alternative way to utilize the crop residues. Thus waste biomass from agricultural production becomes a useful product that supports the physical, chemical and biological conditions in the soil and improves the overall ecological balance (Krishna et al., 2004). The straw is a favorable habitat for the soil- and residue-borne plant pathogens and has high value of C/N - 80:1. According to Khan and Mubeen (2012) wheat straw consists of cellulose (33.7-40%), hemicellulose (21-26%) and lignin (11-22.9%). Therefore, straw decomposition is better to be done by fungi with potential to suppress plant pathogens.

The filamentous fungi of genus Trichoderma are one of the most extensively studied microorganisms, producing cellulolytic enzymes. They are widely used as a biocontrol agents against the soil born phytopathogens and as industrial producers of cellulolytic and hemicellulolytic enzymes (Kubicek et al., 1996; Cumagun et al., 2009; Abo-State et al., 2010; Schuster and Schmoll, 2010).

The aim of this work was to investigate influence of organic nitrogen amendment, containing amino acids on the cellulase and xylanase, produced by *Trichoderma* spp. isolates on wheat straw.

Material and methods

Microorganisms

Fungi of genus *Trichoderma* were isolated from soil samples on Rose Bengal Chloramphenicol Agar (SIGMA-ALDRICH). All 37 local isolates of *Trichoderma* spp. were grown and stored on Potato Dextrose Agar (SIGMA-ALDRICH).

Culture conditions of solid state cultivation

Fungi were grown on wheat straw (5 grams of wheat straw, dried and milled into small pieces (3-9 mm) were mixed with 25 ml distilled water into 500 ml glasses) in two variants: 1. The straw (C:N-80:1), moistened with distillated water; 2. Amended straw (C:N-30:1), adjusted by organic nitrogen amendment, applied as water solution. The glasses were sterilized for 30 min at 121°C, inoculated after cooling with 1 ml spore suspension ($1*10^7$ spores/ml) and incubated at 28°C for 10 days under static condition (Abo-State et al., 2010). Organic nitrogen amendment (ONA) contains 44% of dry matter, 35% of which amino acids and 3.8% total nitrogen.

Enzyme assay

Enzyme extraction. Enzymes were extracted in distilled water (ratio S:W- 1:10) – for 60 min on a rotary shaker (190 rpm) at 28ºC. The solid materials and fungal biomass were separated by centrifugation – 15000 rpm at 4ºC for 30min. The supernatant was used for enzyme analyses. (Abo-State et al., 2010).

Xylanase activity. Xylanase activity was determined by incubating 0.1 ml of culture filtrate with 0.9 mL of 1% Xylan beechwood (SIGMA-ALDRICH) in 0.05 M acetate buffer, pH 5.0 at 50°C for 30 min (Bailey et al., 1992). The reaction was terminated by adding 1 ml of dinitrosalicylic acid (DNSA) reagent and placed in a boiling water bath at 100°C for 5min (Miller, 1959). Absorbance was read at 540 nm on spectrophotometer Spectroquant Prove 300 (Merck). Xylose (SIGMA-ALDRICH) was used as standard. Xylanase activity was expressed as 1 µg of reducing sugar (xylose equivalent) released per minute per milliliter of enzyme solution.

Carboxymethyl cellulase activity. Endoglucanase, Carboxymethyl cellulase (CMCase) activity was measured by determining the amount of reducing sugar released from 1% carboxymethyl cellulose (CMC, SIGMA-ALDRICH). The reaction mixture consisted of 1 mL 1% CMC in 0.1 M Na-acetic buffer, pH 5.0, and 1 mL culture filtrate. After incubation at 63°C for 30 min the reaction was stopped by addition of 2 mL DNSA followed by boiling in a water bath at 100°C for 5 min (Miller, 1959). After cooling the reaction mixture to room temperature, the absorbance values were read at 540 nm on Spectroquant Prove 300. Glucose (SIGMA-ALDRICH) was used as standard. One unit (U) of CMCase activity was expressed as 1 µg of reducing sugar (glucose equivalent) released per minute per milliliter of enzyme solution (Wang et al., 1988).

β-glucosidase activity. The reaction mixture consisting of 1 ml culture filtrate, 4 ml MUB (pH 6.0) and 1 ml 25 mM PNG (p-Nitrophenyl-β-D-glucoside), was incubated at 37°C for 1 h. After incubation 1 ml 0.5 M $CaCl_2$ and 4 ml 0.1 M Tris buffer, pH 12 were added. The absorbance values were read at 400 nm on Spectroquant Prove 300. The unit of β-glucosidase activity is expressed as the amount of released µg of PNP (p-Nitrophenol) per 1h by 1 ml of enzyme (Tabatabai, 1994).

Statistical analysis

All statistical analyses were performed by SPSS statistical software (IBM SPSS Statistics 19.0). The relationships between enzyme activities at different CN ratios were analyzed by Pearson correlation test. A p value of <0.05 was considered statistically significant.

Results and discussion

The improvement of straw biodegradation processes requires necessary knowledge of the factors determining the rate of biodegradation. The nutrients balance, especially carbon to nitrogen ratio, is one of the main factors of the biodegradation. The application of organic fertilizers, containing various forms of organic matter increases. Among them, the amino acid fertilizers are ones, containing the greatest amount of readily assimilable nitrogen. Organic nitrogen amendment (ONA), used in these experiments, is added in an amount, providing a ratio of carbon to nitrogen 30:1. The ability of 37 local isolates of *Trichoderma* spp. to produce cellulases and xylanase were tested in solid state cultivation on wheat straw as a substrate whit two variants – CN 30 and CN 80.

No much research has been done on the ability of *Trichoderma* spp. to produce endoglucanase (CMCase), β-glucosidase and xylanase directly on wheat straw as carbon source and organic nitrogen amendment as nitrogen source. Most studies in this area have been conducted in liquid nutrient media with various sources of carbon and nitrogen (Ja'afaru, 2013; Pandey et al., 2014; Jampala et al., 2017). Crista et al. (2012) have studied the influence of 7 different amino acids on the production of cellulases and xylanases from *Trichoderma reesei* QM-9414 as a nitrogen source by submerged fermentation (of a liquid nutrient medium) in which carbon source is wheat straw. The results demonstrate stimulation of production of this enzymes in the presence in culture medium of asparagine and glutamic acid and inhibition in the presence of methionine.

Except T1T, where the value of 110.2U/ml was measured, endoglucanase activity of the tested isolates, grown on straw, reached 40U/ml at CN80 (Figure 1). The highest activity, from the

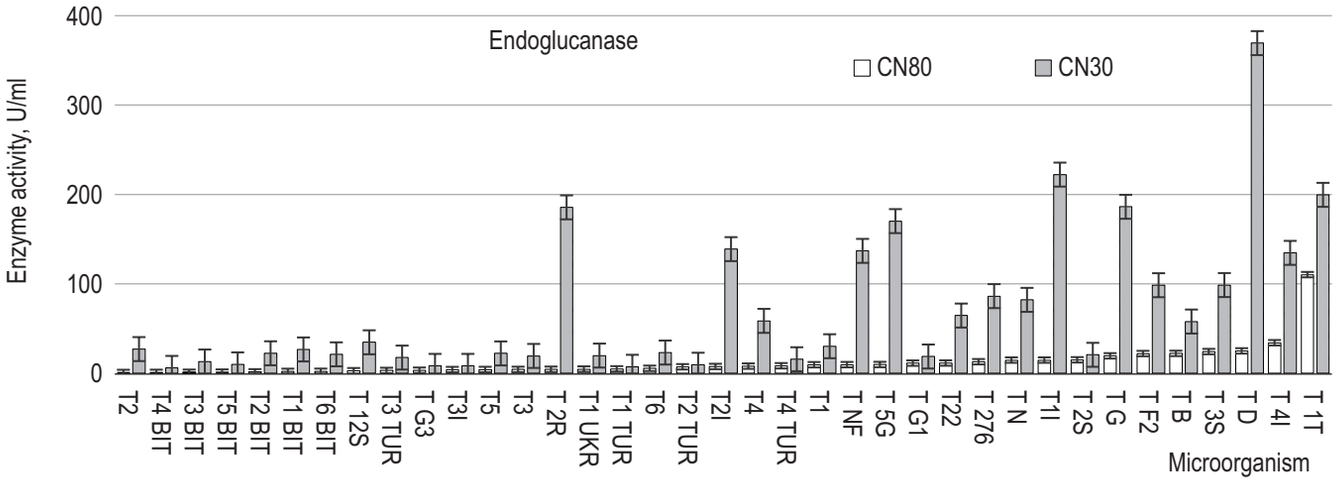

Figure 1. Endoglucanase activities of fungal isolates, grown on a straw (CN80) and on amended straw (CN30)

rest of the tested microorganisms, revealed isolates TG - 19.38, TF2 - 21.95, TB - 22.12, T3S -24.16, TD -24.92 and T4I-34.05U/ml. All other isolates showed activity under 15U/ml, and five of them exhibited no activity on straw at CN80.

The addition of organic nitrogen amendment (ONA) shows a high dependency of the synthesis of the investigated enzymes by the presence of organic nitrogen. There is a multiple increase in the enzyme activity of fungal isolates. The highest activity in presence of ONA was found in isolate TD - 369.1U/ml. Five of the tested isolates (T4I, T22, TNf, TD and T1T) reached the activity over 150U/ml. Three of the evaluated isolates (T1I, T2I and T3I) reached activity between 100 and 150U/ml. The activity of the seven isolates (TG, TG1, TG3, TF2, T2S, T3S and T12S) is between 50 and 100U/ml.

The isolates T12S, TNf, TD, T1I, T5G, T2I, T2 and T2R demonstrated the highest dependency on the availability of ONA. The increase in their activity was up to 42.5 times. For isolate T12S it was 12.5 times, for TNf - 14.3, TD - 14.8, T1I - 15.2, T5G - 17.5, T2I - 18.5 times, while isolates T2 and T2R increased their enzyme activity 34.1 and 42.5 times, respectively.

Abo-State et al. (2010) tested four different agricultural wastes for production of endoglucanase (CMCase) on solid state fermentation and they established that wheat straw is the best substrate for CMCase produced from T. viride – 555U/ml.

The highest β-glucosidase activity at CN80 was observed in TG – 2743.1U/ml (Figure 2). The isolates T4I – 1549.4U/ml, T 2S - 1668.7U/ml, T 3S - 1718.2U/ml and T D - 1824.3U/ml also exhibited high activity. Two isolates, T2I and T1I reached activity of 868.3 and 962.9U/ml, respectively. The other fungi have shown activity below 50U/ml.

Adding ONA greatly enhanced the ability of test microorganisms to synthesize a β-glucosidase. Enormous increase (192.1 times) was observed in isolate T5. The enzyme activity of this isolate was increased from 25.9U/ml in embodiments without an additive to 4975.6U/ml after adding ONA (CN30).

β-glucosidase activity at T2 was increased 68 times - from 72.5 to 4931.9U/ml. Both isolates did not show the highest activity of β-glucosidase after adding of the ONA. The increase in their enzyme production is due to the lower amounts of the enzyme in the absence of additive, which underlines the dependence of its presence.

The highest values of β-glucosidase after adding ONA were recorded at T4 – 10763.1U/ml, and TG – 12679.9U/ml. Both isolates showed higher activity than isolate T5, but the rate of increase in their enzyme activity is less due to the higher values achieved in the culture without the addition of ONA. At T4 enzyme activity increases from 216.6U/ml at CN 80 to 10763.1U/ml at CN 30 (49.7 times). For TG this increase was from 2743.1U/ml to 12679.9U/ml (4.6 times). High enzyme activity was observed in isolates T B – 6474.8, T N – 5557.7, T5 – 4975.6, T2 – 4931.9, T1 – 4785.6 and T 276 –

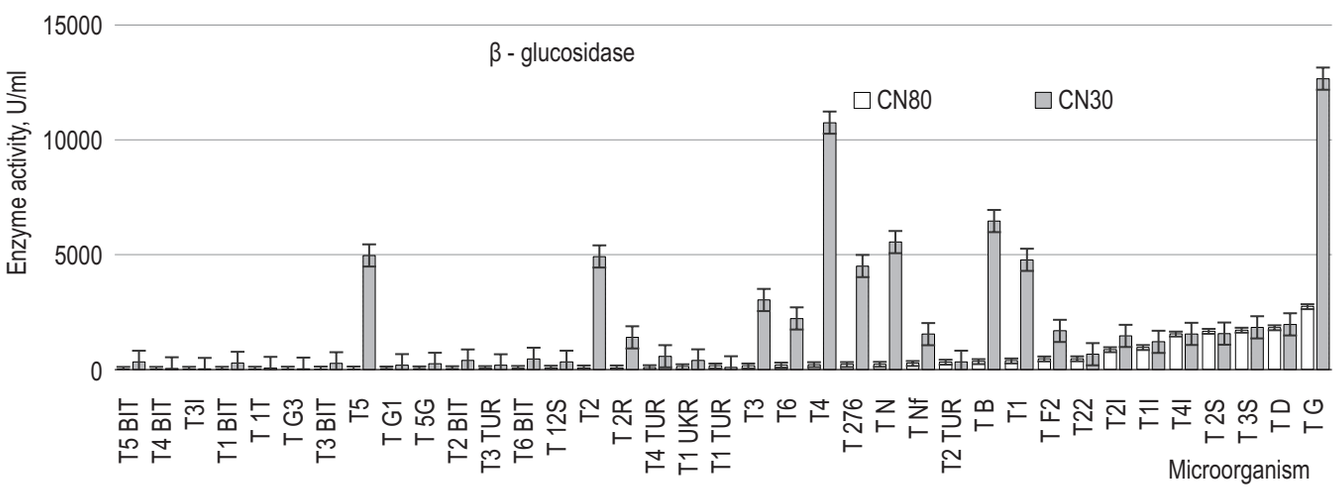

Figure 2. β-glucosidase activities of fungal isolates, grown on a straw (CN 80) and on an amended straw (CN 30)

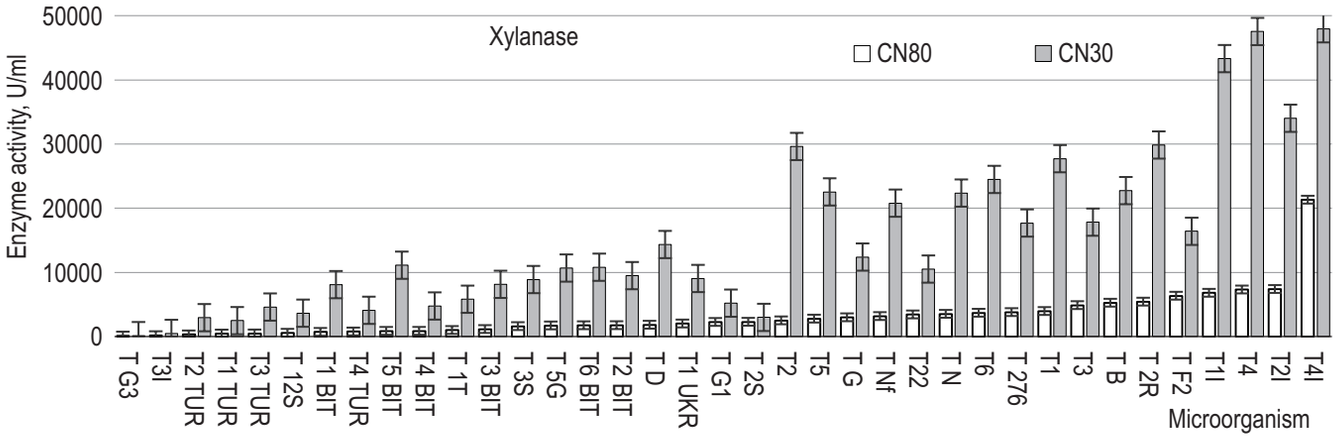

Figure 3. Xylanase activities of fungal isolates, grown on a straw (CN 80) and on an amended straw (CN 30)

4514.3U/ml at CN 30. Eleven isolates (T1I, T4I, T 2R, T2I, T Nf, T 2S, T D, T 3S, T F2, T6, T3) show activities between 1211U/ml and 3034U/ml, and the remaining isolates activity is lower than 1000U/ml.

Xylanase reached the highest activity among tested enzymes at both CN ratios - 80 and 30. (Figure 3). On a native straw (CN 80), nine of all isolates (T1 BIT, T4 BIT, T5 BIT, T1 TUR, T2 TUR, T3 TUR, T4 TUR, T G3, T 12S) demonstrated activity below 1000U/ml. Thirteen of tested isolates reached values from 1000 to 3000U/ml (T2 BIT, T3 BIT, T6 BIT, T1 UKR, T 2, T 5, T G, T G1, T 2S, T 3S, T D, T 1T, T 5G), and another 13 isolates demonstrated xylanase activity above 3000U/ml (T 1, T 3, T 4, T 6, T B, T N, T 276, T F2, T 1I, T 2I, T 22, T Nf, T 2R). An exception was the isolate T 4I, which activity of 21311.5U/ml considerably exceeds the activity of the other isolates at CN 80. The isolate T 3I has not exhibited xylanase activity at CN 80 but at CN 30 it was measured activity 478U/ml.

Again, at CN 30 the xylanase activity was highest for the isolate T 4I – 47937.5U/ml. Sixteen of all isolates (T1 BIT, T2 BIT, T3 BIT, T4 BIT, T1 UKR, T1 TUR, T2 TUR, T3 TUR, T4 TUR, TG1, T2S, T3S, T12S, T3I, T1T, TD) demonstrated activity below 10000U/ml. The other tested isolates showed activity above 10000U/ml. The isolate TG3 demonstrated the lowest enzyme activity after adding the ONA at CN 30.

Ja'afaru (2013) has determined xylanase activity on *T. viride* FD18 – 338U/ml by submerged fermentation whit xylan as carbon source.

On wheat straw all 37 screened local isolates of *Trichoderma* sp. showed different level of enzyme production, both with and without organic nitrogen amendment. Xylanase increases proportionaly to the activity exhibited without the addition of ONA. The increase of β- glucosidase and CMCase does not always follow proportionately the activity exhibited without the addition of ONA (Table 1). In addition, increasing of activity of evaluated enzymes showed different pattern.

Table 1. Pearson's correlation coefficient between changes in enzymatic activity at CN 80 and CN 30

	Control Variables					
	CMC-CN80	CMC-CN30	β-glu-CN80	β-glu-CN30	Xyl-CN80	Xyl-CN30
CMC-CN80	1	0.492**	0.236	-0.007	0.180	0.039
CMC-CN30		1	0.552**	-0.003	0.294	0.355*
β-glu -CN80			1	-0.011	0.346*	0.195
β-glu -CN30				1	0.208	0.402*
Xyl-CN80					1	0.791**
Xyl-CN30						1

Levels of significance at * $p < 0.05$, ** $p < 0.01$.

Comparing the values of the enzyme activities at CN 30 and CN 80 it is obvious that the increase in β-glucosidase is not proportional to the activity displayed on the straw as a single substrate (correlation coefficient, r = -0.011). A moderate positive relationship (r = 0.492 with p <0.01) was observed in endoglucanase activity and a strong uphill linear relationship (r = 0.791 with p <0.01) was found in xylanase activity.

After ONA addition, all enzymes activities have increased several times, indicating the enhancing effect of the additive (Table 2).

Table 2. Average enzyme activity of all tested microorganisms

Enzyme	Enzyme activity at different CN ratio, U/ml		Increase of activity, timesCN30/CN80
	CN80	CN30	
CMCase	11.8	72.1	6.1
β-glucosidase	415.4	2117.9	5.1
Xylanase	3166.9	15535.5	4.9

The average activity of CMCase in all tested isolates increased from 11.8 at CN 80 to 72.1U/ml at CN 30 (6.1 times) The average β-glucosidase activity of all isolates increased 5.1 times (415.4 to 2117.9U/ml), while the xylanase activity increased from 3166.9U/ml at CN 80 to 15535.5U/ml at CN 30 (4.9 times).

Conclusion

There was a significant difference in the enzymatic activity of the isolates in their cultivation on straw with CN 80 and CN 30. The highest carboxymethylcellulose activity at CN 80 showed T1T (110.19U/ml), and in the variant at CN 30 – TD (369.07U/ml) followed by T1I (222.04U/ml). The highest β-glucosidase activity on both variants CN 80 and CN 30 was established for the isolate TG (2743.1U/ml - 12679.9U/ml) and followed by T4 (10763.1U/ml) at CN 30. The highest xylanase activity at CN 80 and CN 30 was measured on the isolate T4I (21311.5U/ml – 47937.5U/ml), followed by T4 (47515U/ml) and T1I (43289.5U/ml) at CN 30. After ONA addition, the average activity of CMCase increased 6.1 times, the average β- glucosidase activity increased 5.1 times, while the xylanase activity increased 4.9 times. A different dependence of activity after addition of ONA, compared to the activity exhibited by fungi, cultured only on straw, was observed. A strong linear relationship was found in the increase of the xylanase activity due to the addition of ONA. The increase in CMCase establishes a moderate relationship and growth of β-glucosidase with no dependence manifested only straw activity.

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Instruction for authors

Preparation of papers

Papers shall be submitted at the editorial office typed on standard typing pages (A4, 30 lines per page, 62 characters per line). The editors recommend up to 15 pages for full research paper (including abstract references, tables, figures and other appendices)

The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods,Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

The title needs to be as concise and informative about the nature of research. It should be written with small letter /bold, 14/ without any abbreviations.

Names and affiliation of authors The names of the authors should be presented from the initials of first names followed by the family names. The complete address and name of the institution should be stated next. The affiliation of authors are designated by different signs. For the author who is going to be corresponding by the editorial board and readers, an E-mail address and telephone number should be presented as footnote on the first page. Corresponding author is indicated with *.

Abstract should be not more than 350 words. It should be clearly stated what new findings have been made in the course of research. Abbreviations and references to authors are inadmissible in the summary. It should be understandable without having read the paper and should be in one paragraph.

Keywords: Up to maximum of 5 keywords should be selected not repeating the title but giving the essence of study.

The introduction must answer the following questions: What is known and what is new on the studied issue? What necessitated the research problem, described in the paper? What is your hypothesis and goal ?

Material and methods: The objects of research, organization of experiments, chemical analyses, statistical and other methods and conditions applied for the experiments should be described in detail. A criterion of sufficient information is to be possible for others to repeat the experiment in order to verify results.

Results are presented in understandable tables and figures, accompanied by the statistical parameters needed for the evaluation. Data from tables and figures should not be repeated in the text. **Tables** should be as simple and as few as possible. Each table should have its own explanatory title and to be typed on a separate page. They should be outside the main body of the text and an indication should be given where it should be inserted.

Figures should be sharp with good contrast and rendition. Graphic materials should be preferred. Photographs to be appropriate for printing. Illustrations are supplied in colour as an exception after special agreement with the editorial board and possible payment of extra costs. The figures are to be each in a single file and their location should be given within the text.

Discussion: The objective of this section is to indicate the scientific significance of the study. By comparing the results and conclusions of other scientists the contribution of the study for expanding or modifying existing knowledge is pointed out clearly and convincingly to the reader.

Conclusion: The most important consequences for the science and practice resulting from the conducted research should be summarized in a few sentences. The conclusions shouldn't be numbered and no new paragraphs be used. Contributions are the core of conclusions.

References:

In the text, references should be cited as follows: single author: Sandberg (2002); two authors: Andersson and Georges (2004); more than two authors: Andersson et al.(2003). When several references are cited simultaneously, they should be ranked by chronological order e.g.: (Sandberg, 2002; Andersson et al., 2003; Andersson and Georges, 2004).

References are arranged alphabetically by the name of the first author. If an author is cited more than once, first his individual publications are given ranked by year, then come publications with one co-author, two co-authors, etc. The names of authors, article and journal titles in the Cyrillic or alphabet different from Latin, should be transliterated into Latin and article titles should be translated into English. The original language of articles and books translated into English is indicated in parenthesis after the bibliographic reference (Bulgarian = Bg, Russian = Ru, Serbian = Sr, if in the Cyrillic, Mongolian = Mo, Greek = Gr, Georgian = Geor., Japanese = Ja, Chinese = Ch, Arabic = Ar, etc.)

The following order in the reference list is recommended:

Journal articles: Author(s) surname and initials, year. Title. Full title of the journal, volume, pages. Example:

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Books: Author(s) surname and initials, year. Title. Edition, name of publisher, place of publication. Example:

Oldenbroek JK, 1999. Genebanks and the conservation of farm animal genetic resources, Second edition. DLO Institute for Animal Science and Health, Netherlands.

Book chapter or conference proceedings: Author(s) surname and initials, year. Title. In: Title of the book or of the proceedings followed by the editor(s), volume, pages. Name of publisher, place of publication. Example:

Mauff G, Pulverer G, Operkuch W, Hummel K and Hidden C, 1995. C3-variants and diverse phenotypes of unconverted and converted C3. In: Provides of the Biological Fluids (ed. H. Peters), vol. 22, 143-165, Pergamon Press. Oxford, UK.

Todorov N and Mitev J, 1995. Effect of level of feeding during dry period, and body condition score on reproductive performance in dairy cows,IXth International Conference on Production Diseases in Farm Animals, September 11–14, Berlin, Germany.

Thesis:

Hristova D, 2013. Investigation on genetic diversity in local sheep breeds using DNA markers. Thesis for PhD, Trakia University, Stara Zagora, Bulgaria, (Bg).

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Animal welfare

Studies performed on experimental animals should be carried out according to internationally recognized guidelines for animal welfare. That should be clearly described in the respective section “Material and methods”.

AGRICULTURAL SCIENCE AND TECHNOLOGY

Volume 9, Number 3
September 2017

Journal web site:
www.agriscitech.eu

Publisher:
www.alfamarket.biz