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C O N T E N T

SINGLE AND SIMULTANEOUS REMOVAL OF As (III) AND PHOSPHATE IONS IN AQUEOUS SOLUTION USING "GREEN" SYNTHESIZED NANO ZERO VALENT IRON (nZVI) FROM GREEN TEA LEAF EXTRACT,
Leovac Maćerak, A., Jokić, J., Kerkez, Đ., Slijepčević, N., Bečelić-Tomin, M., Tomašević Pilipović, D., Dalmacija, B.....8

CNT-BASED DIRECT POTENTIOMETRIC SENSOR FOR DETERMINATION OF ANIONIC SURFACTANTS IN WASTEWATERS NEAR OSIJEK, CROATIA,
Kraševac Sakač, M., Jozanović, M., Sakač, N.....13

IMPROVING NUTRITIONAL EDUCATION IN ROMANIAN SCHOOL,
Cirnatu, D., Ciupe Varga, I., Răducanu, D.....19

SOME WAYS OF CONSTITUTING ECOLOGICAL INTELLIGENCE,
Ćorić, D., Šajn, D.....27

VR IN A FUNCTION OF IMPROVING A PUBLIC URBAN NEEDS,
Šiđanin, P., Plavšić, J.....33

STREET DUST – A CARRIER OF XENOBIOTICS AND A POTENTIAL RISK FACTOR FOR HUMAN HEALTH
Đurišić-Mladenović, N., Škrbić, B., Antić, I., Buljovčić, M., Živančev, J., Tadić, Đ., Marinković, V.....40

PREDICTION OF FATE AND BEHAVIOR OF ILLICIT DRUGS IN WASTEWATER,
Miloradov Vojinović, M., Sremački, M., Dmitrašinović, S.....45

ADAPTATION OF A PLANT GROWTH CHAMBER FOR THE EXPERIMENTAL CULTIVATION OF CHAMPIGNONS (AGARICUS BISPORUS),
Allaga, H., Büchner, R., Hatvai, L., Szekeres, A., Vágvölgyi, Cs., Kredics, L., Manczinger, L.....51

THE ENVIRONMENTAL IMPACTS OF THE OFFSHORE OIL AND GAS INDUSTRY,
Bogdanović, M., Vukićević, M.....60

HEALTH CARE STAFF PERCEPTION REGARDING RADIOPROTECTION,
Cirnațu, D., Bursuc, A.....69

ECOLOGICAL AND HUMAN HEALTH RISK ASSESSMENT OF HEAVY ELEMENTS IN STREET DUST IN TIANJIN, CHINA,
Živančev, J., Ji, Y., Škrbić, B., Antić, I., Buljovčić, M.....75

EXTRACELLULAR ENZYME ACTIVITIES OF CLINICAL AND ENVIRONMENTAL FUSARIUM SOLANI SPECIES COMPLEX ISOLATES,
Homa, M., Galgóczy, L., Kredics, L., Manikandan, P., Shobana, C.S., Vágvölgyi, Cs., Papp, T.....82

NON-ALCOHOLIC FATTY LIVER DISEASE AND EFFECTS OF LIFESTYLE MODIFICATION,
Iova, C., Zorila, C., Mos, L.....87

INVESTIGATION OF THE CAROTENE DYESTUFF MATERIALS AND CAPSAICIN CONTENT OF DIFFERENT SPECIAL SPICE RED PEPPER VARIETIES AND THEIR ALTERNATIVE USAGE,
Lantos, F., Hajdu, M., Yildirim, I.....92

DETERMINATION OF CARCINOGENIC COMPOUNDS OF THE JAPANESE ASHITABA (ANGELICA KEISKEI KOIDZUMI ITO.) HERB,
Lantos, F., Papp, Z.....96

THE EFFECTS OF VARIOUS NUTRIENT SOURCES ON THE YIELD AND MARKETABILITY OF SWEET POTATO [IPOMOEA BATATAS (L.) LAM.],
Pap, L., Szarvas, A., Hódi Szél, M., Monostori, T.....102

SOCIAL MEDIA HABITS AND ADDICTION OF MEDICAL STUDENTS,
Parscuta, D., Farcas, R., Farcas, E., Cirnatu, D., Ursoniu, S.....107

CHARACTERISATION OF MICROPOROUS BIOCHAR SYNTHESISED FROM LIGNOCELLULOSIC WASTE BIOMASS,
Paunović, O., Pap, S., Radović, S.,Turk Sekulić, M.....114

PERSONALITY PROFILES AND BODY MASS INDEX RELATED TO GENDER AND AGE GROUPS IN A POPULATION OF MEDICAL STUDENTS: A CASE STUDY,
Petrescu, C.....120

APPLICATION OF THE MODIFIED CARBON PASTE ELECTRODES FOR DETERMINATION OF CATECHIN,
Stojanović, Z., Đurović, A., Kravić, S., Suturović, Z., Brezo, T., Richtera, L., Bytešníková, Z., Grahovac, N.....126

PHOSPHATES FROM DETERGENTS – PROBLEMS AND SOLUTIONS,
Šćiban, M., Kukić, D., Vasić, V., Prodanović, J., Prorok, V.....130

LEVEL OF HEAVY ELEMENTS IN VEGETABLES FROM FLOODED ARABLE SOIL IN VOJVODINA PROVINCE: HEALTH RISK ASSESSMENT,
Škrbić, B., Antić, I., Živančev, J., Buljovčić, M., Johansen, J.E., Stojanović, G.....136

CONTAMINATION AND HEALTH RISK ASSESSMENT OF PAHS IN FARMLAND SOILS,
Škrbić, B., Antić, I., Živančev, J., Đurišić-Mladenović, N., Vágvölgyi, Cs.....146

ASSESSMENT OF HEAVY ELEMENT LEVELS IN AGRICULTURAL SOIL FROM HUNGARY-SERBIA BORDER REGION,
Škrbić, B., Buljovčić, M., Panković, D., Vágvölgyi, Cs.....153

EVALUATION OF HEAVY ELEMENTS IMPACT ON ENVIROMENT IN SOIL SAMPLES USING POLLUTION INDICES,
Škrbić, B., Buljovčić, M.....161

POLYCYCLIC AROMATIC HYDROCARBONS IN STREET DUST OF NOVI SAD, SERBIA: LEVELS AND RISKS,
Škrbić, B., Đurišić-Mladenović, N., Živančev, J., Tadić, Đ.....167

EXOENZYME PRODUCTION OF ENDOPHYTIC FILAMENTOUS FUNGI DERIVED FROM COMMON YEW (TAXUS BACCATA) SAMPLES,
Volford, B., Szekeres, A, Papp T., Papp, Zs., Škrbić, B., Mondal, K.C., Vágvölgyi, Cs., Takó, M.....174

WHETHER THE FERTILIZATION OF AGRICULTURAL SOIL WITH LIVESTOCK MANURE MAY TRIGGER OFF ADVERSE EFFECTS ON HUMAN HEALTH?,
Antić, I. , Matamoros, V., Škrbić, B.D., Bayona, J.M.....180

Authors' index.....186

EXOENZYME PRODUCTION OF ENDOPHYTIC FILAMENTOUS FUNGI DERIVED FROM COMMON YEW (*TAXUS BACCATA*) SAMPLES

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Abstract
The fungal endophytes reside in living plant tissues often in an asymptomatic host-fungus interaction. Recently, a variety of endophytic fungi have been documented as producers of plant material degrading exoenzymes. The secreted enzymes are useful in the invading and colonization processes and help the fungus to obtain monomer sugars as nutrients from the environment. These hydrolases and/or the producing microorganisms can be utilized for biotechnological applications. Despite the high-yield hydrolase producing potential, the endophytic fungi are little explored in this regard.
Key Words: endophytic fungi; exoenzyme activities; hydrolases; screening

Introduction

Fungal endophytes are a very important and diverse group of microorganisms. They can be found in different part of plants such as bark, leaves and root (Somjaipeng et al., 2015; Kaul et al., 2012). Healthy plant tissues are colonized by endophytic fungi without causing any overt symptoms in or apparent injury to the host. In many cases, this relationship could be beneficial to the host as well (Kaul et al., 2012; Pimentel et al., 2011; Staniek et al., 2008; Kusari and Spiteller, 2012; Ibrahim et al., 2018). Several representatives of endophytic fungi are known as good producers of bioactive substances, including medicinal products, which can be useful in treatment of various diseases and microbial infections (Pimentel et al., 2011; Kusari et al., 2012; Katoch et al., 2014). Furthermore, the fungal endophytes produce plant degrading exoenzymes, e.g., cellulases, pectinases, amylases and xylanases, that help them to invade and colonize the host plant tissue and to obtain nutrients from their environment. From practical point of view, these enzyme activities can be utilized in various biotechnological applications, such as in the food, pharmaceutical and biofuel production industries (Silvério et al., 2018; Almeida et al., 2011). For instance, the cellulolytic and xylanolytic cocktails obtained from these organisms could be very important supports in the complete saccharification of lignocellulosic materials to fermentable sugars. Although there are many reports on the production of industrial enzymes from different microbial sources, less attention has been paid on the fungal endophytes in this regard.
In the present study, we aimed to evaluate the exoenzyme production of fifteen fungal endophytes, i.e. *Neofusicoccum* (1), *Mucor* (5), *Aspergillus* (1), *Sordaria* (1), *Fusarium* (2), *Phoma* (1), *Penicillium* (2) and *Trichoderma* (2) isolates obtained from *Taxus baccata* samples, and deposited in the Szeged Microbiological Collection (SZMC) prior to this research (Volford, 2016). The study included the investigation of their cellulase, xylanase, amylase, lipase and beta-galactosidase activities using conventional plate screening assays.

Materials and methods

Beta-galactosidase activity detection

An X-gal-based simple and rapid method was used to screen the beta-galactosidase production in

Petri-dishes. The applied medium contained the following components (% w/v): malt extract (2), lactose (2), peptone (0.1), agar (2), and X-gal (20 mg/ml in dimethyl-sulfoxide). A volume of 20 µl of spore suspension (1 x 10^6 spore/ml) was added to each agar plates and the cultures were incubated for ten days at optimal growth temperature of fungi. Growth and enzyme production of fungi were detected visually every day. Isolates with beta-galactosidase activity provided deep blue colored plates (Silvério et al., 2018).

Amylase activity detection

We used a conventional starch agar protocol to test the selected endophytes for amylase production. In this screening procedure, a volume of 20 µl spore suspension (1 x 10^6 spore/ml) was pipetted to the middle of Petri-dishes contained the following medium (% w/v): soluble starch (10), yeast extract (5), and agar (3). The plates were kept at optimal temperature for growth for three days. The growth was checked daily and after the three-days incubation period, the enzyme production was visualized by adding potassium iodide solution (1 ml/l).

Cellulolytic activity detection

Cellulolytic activity of the selected endophytes was screened through the hydrolysis of carboxymethyl cellulose (CMC). The medium used for this analysis contained (% w/v): CMC (4), mannitol (1), KH_2PO_4 (0.5), $NaNO_3$ (0.2), $MgSO_4$ (0.1), agar (1.7) and digitonin (5 µg/ml). As in the previously described procedures, a drop of 20 µl spore suspension (1 x 10^6 spore/ml) was pipetted onto the surface of the agar plates which were incubated for three days before evaluation. Enzyme production was detected by staining the plates with Congo red solution (1 mg/ml) for one hour (10 ml/Petri plates).

Xylanase activity detection

Xylanase activity screening tests were performed with xylan supplemented medium. The solid medium (mannitol, 1%; KH_2PO_4, 0.5%; $NaNO_3$, 0.2%; $MgSO_4$, 0.1%; agar 1.7; digitonin 5 µg/ml; xylan, 4%) was prepared in Petri-dishes and inoculated with spore suspensions as described above. These were incubated for three days at temperatures appropriate for the growth of the tested fungus. Similar to CMC hydrolysis assay, enzyme production was detected with Congo red solution (10 ml/Petri plates from 1 mg/ml solution). After one-hour incubation with the dye, a clear activity zone was formed around the fungi that possess high enzyme activity.

Lipase activity detection

For lipase activity screening, we used the following medium (% w/v): yeast extract (0.6), peptone (1) and agar (1). After sterilization and cooling, 0.5% tributyrin was added to the medium, then, it was shaken intensively and poured into the Petri-dishes. After inoculation, the cultures were grown for three days, and the activity was evaluated from the size of the clearing zone around the colonies.

Results and discussion

Beta-galactosidase production

The most promising beta-galactosidase producers were the *Trichoderma harzianum* SZMC 24022 and the *Penicillium chrysogenum* SZMC 24018 isolates (Table 1). Furthermore, *Fusarium sp.* SZMC 24800, *Neofusicoccum parvum* SZMC 24782 and *Sordaria sp.* SZMC 24777 can also be potential producers of beta-galactosidase. There was no detectable activity in case of the *Mucor sp.* SZMC 24852, SZMC 24853, SZMC 24873, SZMC 24875 and SZMC 24916, *Aspergillus sp.*

SZMC 24801, *Fusarium lateritium* SZMC 24783, *Phoma fungicola* SZMC 24779 and *Penicillium hordei* SZMC 24799 isolates.

Table 1. Beta-galactosidase activity of the tested fungal endophytes. The intensity of the blue color (0-5 index) is proportional with the enzyme activity.

Fungal strains	SZMC codes[a]	0th day	2nd day	4th day	6th day	8th day	10th day
Neofusicoccum parvum	SZMC 24782	0[b]	0	0	5	5	5
Sordaria sp.	SZMC 24777	0	0	0	3	4	5
Fusarium sp.	SZMC 24800	0	0	0	4	5	5
Penicillium chrysogenum	SZMC 24018	0	1	2	5	5	5
Trichoderma atroviride	SZMC 23992	0	0	0	2	4	5
Trichoderma harzianum	SZMC 24022	0	1	2	5	5	5

[a] SZMC: Szeged Microbiology Collection
[b] 0: no color; 1: light blue; 2: darker blue; 3: blue; 4: dark blue; 5: deep dark blue

Amylase production

After evaulating the results of the amylase activity test, four strains, i.e. the *Fusarium sp.* SZMC 24800, *N. parvum* SZMC 24782, *T. atroviride* SZMC 23992 and *T. harzianum* SZMC 24022 were found as the best producers (Figure 1).

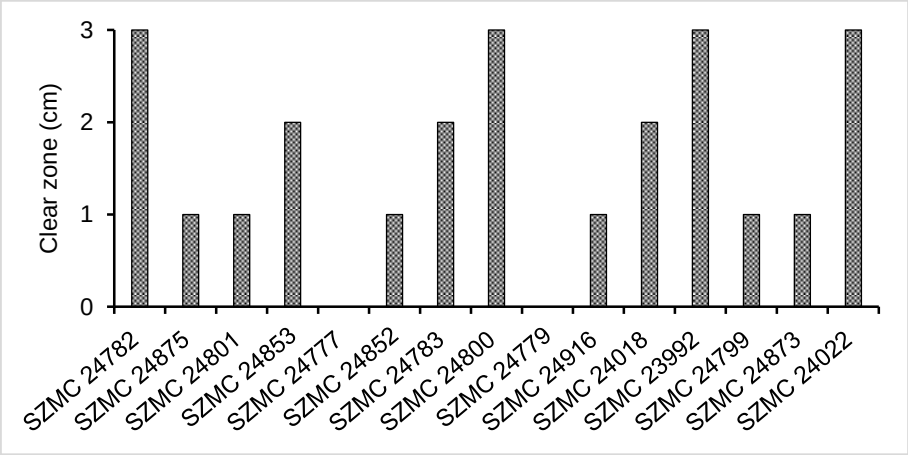

Figure 1. Detection of amylase activity on starch agar.

Cellulolytic enzymes production

Cellulase activity of the fungal endophytes was monitored on medium containing CMC. In these test, five fungal strains were selected as good cellulase producers. *Fusarium lateritium* SZMC 24783, *Sordaria sp*. SZMC 24777, *Penicillium hordei* SZMC 24799 and *Mucor sp.* SZMC 24852, SZMC 24916 strains exhibited the highest CMC hydrolyzing activity (Figure 2).

Xylanase activity

In xylanase production tests, only few isolates showed noticeable enzyme activity. The overall xylanase producing ability of the tested strains was low as compared to the other enzymes studied.

Under the applied growth condition, the *Mucor sp.* SZMC 24852 strain proved to be the best xylanase producer fungal strain (Figure 3).

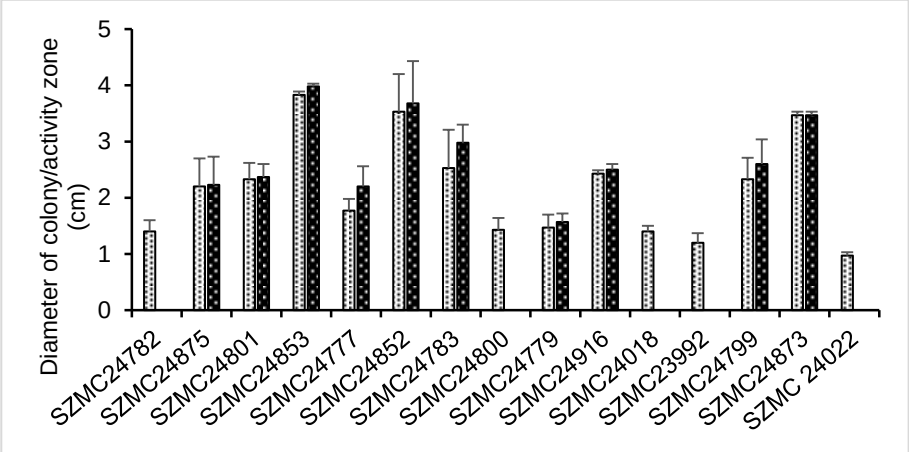

Figure 2. Detection of cellulolytic activity of the tested fungal endophytes on CMC agar. Light gray column: diameter of the fungal colony; Dark gray column: diameter of the activity zone.

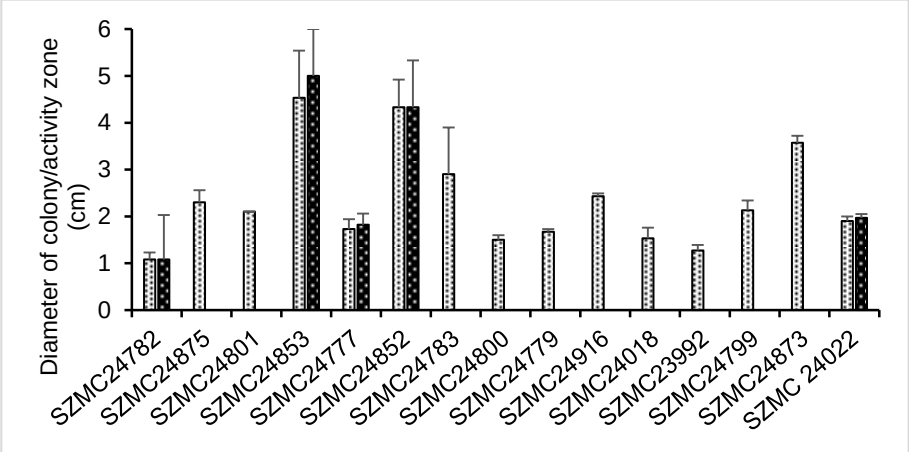

Figure 3. Detection of xylanase activity of the tested fungal endophytes on xylan containing medium. Light gray column: diameter of the fungal colony; Dark gray column: diameter of the activity zone.

Lipase activity

Concerning lipase production, many fungal strains showed extracellular lipolytic activity towards tributyrin. Under the screening condition applied, the *Mucor sp.* SZMC 24852, SZMC 24853, SZMC 24873 and SZMC 24875, *Aspergillus sp.* SZMC 24801, *T. harzianum* SZMC 24022, *Ph. fungicola* SZMC 24779, and *Sordaria sp.* SZMC 24777 isolates demonstrated the highest lipase activity (Table 2).

Table 2. Lipolytic activity of the isolated fungal endophytes.

Fungal strains	SZMC codes	Activity	No activity
Neofusicoccum parvum	SZMC 24782	-	X
Mucor sp.	SZMC 24875	X	-
Aspergillus sp.	SZMC 24801	X	-
Mucor sp.	SZMC 24853	X	-
Sordaria sp.	SZMC 24777	X	-
Mucor sp.	SZMC 24852	X	-
Fusarium lateritium	SZMC 24783	-	X
Fusarium sp.	SZMC 24800	-	X
Phoma fungicola	SZMC 24779	X	-
Mucor sp.	SZMC 24916	-	X
Penicillium chrysogenum	SZMC 24018	-	X
Trichoderma atroviride	SZMC 23992	-	X
Penicillium hordei	SZMC 24799	-	X
Mucor sp.	SZMC 24873	X	-
Trichoderma harzianum	SZMC 24022	X	-

X: activity
- : no activity

Conclusion

In this study, extracellular hydrolase activities of fungal endophyte isolates obtained from *T. baccata* were investigated using conventional agar plate methods. After the incubation and appropriate staining, results revealed a high variability in the enzyme production of the tested strains. Majority of the isolates exhibited considerable amylase and cellulase activities. Remarkable beta-galactosidase activity has been identified for *Trichoderma sp.*, *N. parvum*, *Fusarium sp*., *Sordaria sp.* and *P. chrysogenum* isolates. The best producers with a wide range of enzyme activities were *Sordaria sp., T. harzianum, Mucor sp.* and *Aspergillus sp.* isolates. These fungi and the produced enzymes seem to be potential candidates for future industrial applications.
In conclusion, the results presented here would be a good basis for the selection of novel exoenzyme producer strains. These could support further basic and applied research. However, additional liquid and solid-state fermentation studies are needed to optimize the conditions of the high-yield production.

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