JJBS

Volume 15, Number 3,September 2022
ISSN 1995-6673
Pages 457 – 461
https://doi.org/10.54319/jjbs/150315

Jordan Journal of Biological Sciences

Biodegradation of Doxycycline Hyclate by Local *Purpureocillium lilacinum* strain PlHN17 and *Trichoderma asperellum* isolate Tullur: Monitoring by Antimicrobial Activity

Fathin Fashihah Kamaruddin[1], Maslinda Musa[1], Mohd Muzamir Mahat[1], Shahrul Hisham Zainal Ariffin[2], Muhd Fauzi Safian[1] and Zaidah Zainal Ariffin[1*]

[1]*Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, UiTM, Malaysia;* [2]*School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia*

Received: September 9, 2021; Revised: January 11, 2022; Accepted: January 19, 2022

Abstract

In this study, two soil fungi from ruminant farms soil that has been exposed to tetracycline antibiotic with GPS coordinate 2°59'28.8"N 101°44'00.5"E were isolated and identified. Identification was carried out based on morphological observations, macroscopic, microscopic and molecular identification, polymerase chain reactions (PCR) and sequencing. The isolated species were identified as *Purpureocillium lilacinum* strain PlHN17 (S1) and *Trichoderma asperellum* isolate Tullur (S12). Growth profiles for each of the isolated fungi were constructed and the fungi showed exponential phase from day 4 to day 5. Both fungi went through a biodegradation process and antimicrobial activity of doxycycline hyclate antibiotic residues was determined. The finding revealed *P. lilacinum* strain PlHN17 (S1) has the highest deactivation ability of doxycycline hyclate antibiotic (77.78 %) compared to *T. asperellum* isolate Tullur (75.45 %). Hence, the isolated *P. lilacinum* strain PlHN17 (S1) showed the potential as a bioremediation tool to deactivate the doxycycline hyclate antibiotic.

Keywords: Biodegradation, Doxycycline Hyclate, *Purpureocillium lilacinum* strain PlHN17, *Trichoderma asperellum* isolate Tullur

1. Introduction

Tetracyclines possess many benefits for instance a wide range of antimicrobial activity, low toxicity, and mostly oral administration. The beneficial antimicrobial properties of these antibiotics have contributed to their extensive use in therapy and prophylaxis of human infections along with the absence of major adverse side effects (Chopra and Roberts 2001; Eliopoulos *et al.* 2003). Doxycycline hyclate (DOXH) is a hydrochloride hemiethanol hemihydrate of doxycycline (DOX) with a molecular mass and molecular formula of 512.94 g/mol. It is more frequently used in pharmaceutical samples as it is much more soluble compared to doxycycline monohydrate. DOXH is a semisynthetic broad-spectrum tetracycline antibiotic, and it is widely used in veterinary medicine. It is also used as an animal feed supplement to prevent diseases (Kogawa and Salgado, 2013). DOXH is more active than tetracycline against many bacterial species such as *Streptococcus pyogenes*, enterococci, *Nocardia* spp and several anaerobic species. In comparison with other tetracyclines, DOXH is preferable in the treatment of certain infections such as chronic prostatitis, sinusitis, syphilis, chlamydia and pelvic inflammatory disease due to better absorption and prolonged half-life thus permitting less persistent dosage (Kogawa and Salgado, 2013). However, the increase in the use of antibiotics such as tetracyclines especially DOXH for veterinary and medical purposes, affects the environment and human health as active antibiotics forms are excreted from the body via urine and/or feces into the environment (Javid *et al.* 2016). As a result, the overuse of these antibiotics may pollute water resources.

Tetracyclines in water and soil can cause allergies and toxicity as these compounds remain active. For example, excreted antibiotics affect almost all bacterial species in the environment and cause these species to develop resistance to these compounds (Pena *et al.* 2010). Tetracyclines are classified as the most detected antibiotics in sewage, domestic wastewater, drinking water, sludge, surface and groundwater resources. The reasons behind this are the increased usage of tetracyclines and the inefficiency to remove tetracyclines by most conventional wastewater treatment processes, which is why tetracycline is now more likely to pollute surface and groundwater resources (Javid *et al.* 2016).

The biodegradation process is crucial for overcoming the inefficiency of most conventional wastewater treatment processes in the removal of tetracycline especially DOXH. Filamentous fungi are versatile and robust organisms with enormous capabilities for biodegradation of several substrates and therefore are preferable (Carvalho *et al.* 2010). The contamination of DOXH in the soil surface and groundwater resources can be prevented by carrying out the fungal biodegradation process.

* Corresponding author. e-mail: drzaidah@uitm.edu.my.

2. Materials and Methods

2.1. 2.1 Soil Sample Collection

Three soil samples with triplicates were collected randomly from different sites within the depth of 20 cm at Serdang with GPS coordinate 2°59'28.8"N 101°44'00.5"E where veterinary antibiotics have been used extensively. Different sites were chosen for sample collection to obtain a diverse soil fungal cultures that have been exposed to the veterinary antibiotics. The sites were the dairy cattle farm, beef cattle farm and sheep farm. A soil sample (500 g) was collected and stored in sterile plastic bags for the analysis of pH, temperature and moisture at the laboratory.

2.2. Isolation of Soil Fungi

The soil samples were serially diluted. A ten folds serial dilution was prepared from the microbial suspension. Soil with 3 and 4 dilution factors were plated on Potato Dextrose Agar (PDA) (Merck, Germany). Afterward, the PDA plates were incubated for 3 to 5 days at 28 °C. A single colony was streaked on PDA plates in order to obtain pure cultures. The pure cultures were labelled S1 and S12 respectively. The plates were incubated for 3 to 5 days and stored at 4 °C (Muthulakshmi *et al*. 2011).

2.3. Identification of Isolated Soil Fungi

The isolated soil fungi were established by morphological observations; macroscopic and microscopic. The growth, pigment production, presence of mycelium and the colony characteristics were determined via naked eye observation for macroscopic identification (Kim *et al.* 2011). The stained fungi were viewed under a light microscope (Olympus, CX12) using 1000X magnification for hyphae and conidia observation for microscopic identification.

2.4. 2.4 DNA Extraction and Amplification

Molecular identification of the isolated fungi was identified based on Mohammad *et al*. (2018).

2.5. Growth Profile of Isolated Fungi

The growth profile was determined based on the dry weight of the fungus. During the growth of strains in Potato Dextrose Broth (PDB) (Merck, Germany), a volume of 6 mL was withdrawn at 5 different times (48, 96, 144, 192, 240 h). The mycelia were filtered on a Whatman 125 mm filter paper and their dry cells weights were determined after drying at 70 °C for 24 hours. The growth profiles of fungi were constructed from the dry cell weights (g) plotted versus incubation time (h). The experiments were conducted in triplicate (Melgar *et al*. 2013, Mohammad *et al.* 2018).

2.6. Biodegradation of Doxycycline Hyclate Antibiotic

Four of 250 mL Erlenmeyer flasks containing 100 mL of Potato Dextrose Broth (PDB) were prepared and sterilized using autoclave. Approximately $1x10^6$ CFU/mL of spore suspension solution of the isolated fungi was inoculated into each culture flask respectively. A flask containing PDB and culture only was used as biotic control whereas a flask containing PDB with doxycycline hyclate only was used as abiotic control. All culture flasks were incubated at 28 °C on a rotary shaker at 120 rpm until the mid-exponential phase was reached. All experiments were conducted in triplicate. After the exponential phase, a volume of 10 mL of doxycycline hyclate antibiotic (Sigma-Aldrich, USA) (final concentration 500 µg/mL) was added into each culture flask. The flasks were further incubated at 28 °C for another 96 hours on the rotary shaker at 120 rpm (C˘vanc˘arová *et al.* 2015). A volume of 6 mL of medium was collected as a biodegradation product. The collection of biodegradation product was carried out every 24 hours until 96 hours of incubation was achieved (Zikmundová *et al.* 2002). All biodegradation products and controls were proceeded for the extraction of doxycycline hyclate antibiotic residual.

2.7. Liquid-Liquid Extraction (LLE)

The biodegradation products were subjected to liquid-liquid extraction (LLE) every 24 hours of incubation time. A volume of 6 ml of sample was mixed with 40 mL of extraction solution, 0.1 M of McIlvaine buffer-EDTA and vortexed for 60 seconds before placing it into an ultrasonic bath for 10 minutes. A volume of 10 ml of ethyl acetate: n-heptane (50:50) was added into the tube. The tube was shaken for 2 minutes to create two layers and left for 10 minutes. A volume of 1 ml of the organic phase supernatant was transferred into a microcentrifuge tube and dried using Hypervac vacuum concentrator (VC2200) (Gyrozen, Korea).

2.8. Residual Antimicrobial Activity Assay of Doxycycline Hyclate

Antimicrobial assay was performed to evaluate the activity of doxycycline hyclate antibiotic residual after biodegradation process. *Bacillus subtilis* strain ATCC6633 which is a Gram-positive bacterial strain and susceptible to tetracyclines was used for antimicrobial activity assay. The antimicrobial assay was carried out by using well diffusion techniques. The bacteria were grown in Mueller-Hinton Broth (MHB) (Oxoid, UK) overnight and diluted to 10^8 cfu/mL based on 0.5 McFarland turbidity standard. Doxycycline hyclate antibiotic (30 µg/mL) as a positive control and 70 % ethanol and sterile DMSO as negative controls. The plates were incubated overnight at 37 °C. The zones of inhibition produced by each well were measured and recorded. This assay was done in triplicate (Mohammad *et al.* 2018).

3. Results and Discussion

3.1. Identification of Isolated Fungus

Two fungal species were successfully isolated from the soil that had been exposed to veterinary antibiotics. The species were identified by morphological and molecular identifications as *P. lilacinum* strain PlHN17 (Isolate S1) and *T. asperellum* isolate Tullur (Isolate S12). Morphologically, the reproductive characteristics can be observed from the 1000X magnification. Isolate S1 (Figure 1) has similar microscopic images with the one reported by de Sequeira *et al.* (2017) which resembled *P. lilacinum.* Conidiophores verticillate with two or four phialides which have a swollen basal portion tapering into a short distinct neck. Meanwhile, the conidia are hyaline and ellipsoidal to fusiform (de Sequeira *et al*. 2017).

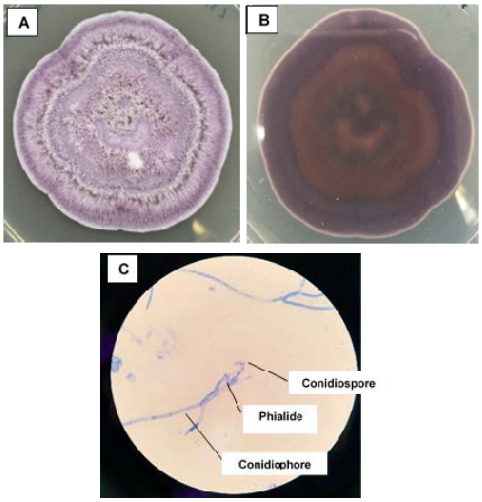

Figure 1. Macroscopic observations of isolate S1 after 5 days of incubation (A: Front view; B: Reverse view) and microscopic observation of isolate S1 under 1000X total magnification (C) using light microscope (Olympus, CX12)

The macroconidia of isolate S12 are oval, large and cumulative over the conidiophores as reported by Samuels *et al.* (1999) that resembled *T. asperellum* (Figure 2). The isolated fungal species were from two different genera, and both species were normally found in soil.

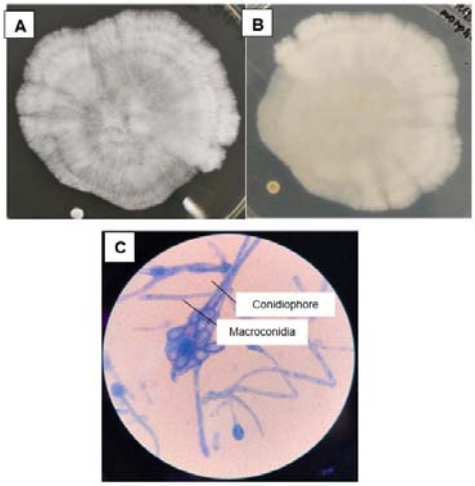

Figure 2. Macroscopic observations of isolate S12 after 5 days of incubation (A: Front view; B: Reverse view) and microscopic observation of isolate S12 under 1000X total magnification (C) using light microscope (Olympus, CX12)

The molecular identification was carried out by DNA barcoding using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases by using NCBI-BLAST. Sequence analysis of the ITS regions of the nuclear encoded rDNA revealed significant alignments of 100% with the isolated fungal species. According to NCBI-BLAST, *P. lilacinum* strain PlHN17 and *T. asperellum* isolate Tullur have query length of 571 and 585 base pairs respectively (NCBI 2019). The query length of both isolates was similar to the finding from the agarose gel electrophoresis conducted which revealed that both isolates relatively are 600 base pairs in length (Figure 3, Table 1)

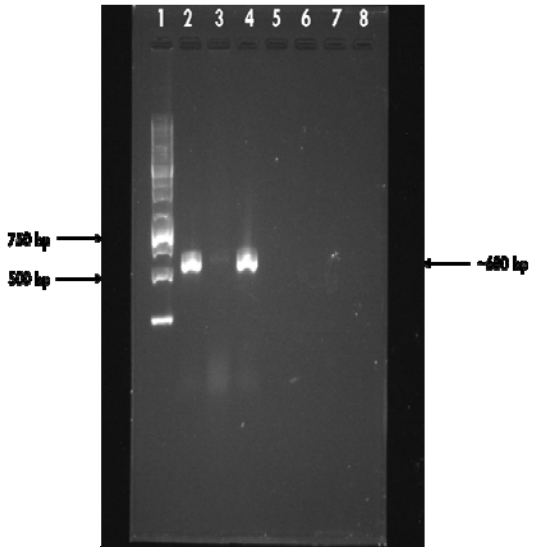

Figure 3. Agarose gel electrophoresis (1%) of genomic DNA of the fungi isolates (S1 and S12) amplified with ITS1 and ITS4 primers. Lane 1: 1kb DNA ladder marker (Solis Biodyne), Lane 2: Isolate S1 and Lane 4: Isolate S12.

Table 1. Summary of NCBI Blast of the isolated fungi

Isolate	Description	Max Score	Total Score	Query Cover	E Value	Identity	Accession
S1	*Purpureocillium lilacinum* strain PlHN17 internal transcribed spacer 1, partial sequence	889	889	81%	0.0	100%	MH483732.1
S12	*Trichoderma asperellum* isolate Tullur internal transcribed spacer 1, partial sequence	837	903	87%	0.0	100%	MN396441.1

3.2. Growth Profile of The Identified Soil Fungi

The growth profiles of two isolated fungal species were constructed (Figure 4). Based on the graph plotted, the exponential or log phase of the isolated fungi occurred from day 4. *P. lilacinum* strain PlHN17 showed exponential growth on day 4 of incubation and the stationary phase began on day 6 in PDB which is faster than in Karanja deoiled cake liquid medium (Sharma *et al.* 2016). *T. asperellum* isolate Tullur was an intermediate growing fungus since the exponential phase occurred on day 1 to day 4 of incubation and experienced stationary phase on day 5 which is agreeable with Saranya *et al.* (2020).

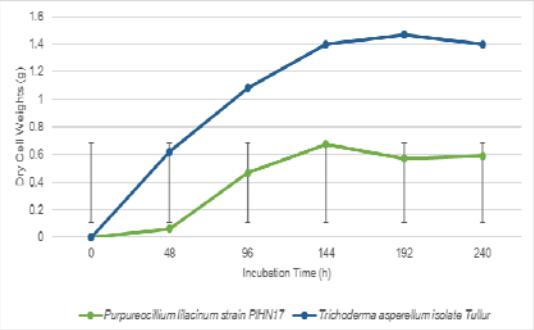

Figure 4. Growth profiles of the isolated fungal species in triplicates after 10 days of incubation.

3.3. Antimicrobial Activity of Doxycycline Hyclate Residues

Both isolated fungal species were subjected to undergo the biodegradation assay of doxycycline hyclate antibiotic. Thus, the ability of fungi to degrade antibiotics, doxycycline hyclate in this case, could be better studied as different fungal strains were being tested. The percentage of inhibition was calculated by dividing the inhibition zone diameter of the doxycycline hyclate residues with the inhibition zone diameter of the abiotic control. The diameter of abiotic control served as a control in which no degradation of doxycycline hyclate antibiotic occurred. Both isolated fungal species exhibited good degradation activity as the percentage of inhibition of doxycycline hyclate antibiotic residues against *Bacillus subtilis* strain ATCC6633 decreased after 96 hours of incubation (Figure 5).

Generally, doxycycline hyclate went through epimerization and this reaction can occur at C-4 and C-6 positions thus resulting in degradation products which are 4-epidoxycycline (4-EDOX), 4,6-epidoxycycline (4,6-EDOX) and 2-acetyl-2-decarboxiamidodoxycycline (ADDOX) (de Barros *et al.* 2018). Genera *Purpureocillium* has the ability of parasitizing fungi, nematodes, and arthropods by enzymatic penetration through cellulase, glucanase, laccase, leucinoxin, lipase, pectinase, protease, chitinase or xylanase release which are involved in the infection process (Moreno-Gavíra 2020). Suda *et al.* (2012) reported that the laccase-HBT system facilitated the elimination of doxycycline as it was completely eliminated after 0.25 h. A study by Xie *et al.* (2016) observed the production of proteolytic enzymes and carbohydrate hydrolases (Xie *et al.* 2016) such as chitinases and serine proteases by *P. lilacinum*. Moreover, *P. lilacinum* has the ability to synthesize heterogenous biologically active secondary metabolites including polyketides and non-ribosome-synthesized peptides (Xie *et al.* 2016).

In our finding, *T. asperellum* isolate Tullur showed a 75.45 % degradation of doxycycline hyclate antibiotic. *T. asperellum* strain BPLMBT1 was reported to produce laccase with excellent temperature and pH stabilities (Shanmugam *et al.* 2020). Research by Zafra et al. (2015) observed a 78 % of phenanthrene degradation by *T. asperellum* after 14 days in soil contaminated with 1000 mg kg^{-1} of phenanthrene. The results of the research revealed the potential of *T. asperellum* to be used in a bioremediation process and served as evidence that *T. asperellum* was considered a promising and effective PAH-degrading microorganism (Zafra *et al.* 2015).

In this study, *P. lilacinum* strain PlHN17 exhibited the highest degradation ability (77.78 %) to degrade doxycycline hyclate (Figure 5). With a prolonged incubation period, doxycycline hyclate antibiotic could be completely removed due to its rapid growth that leads to a high level of mycelia mass production and therefore high level of degradation. To date, no research has been conducted to study the degradation or transformation of doxycycline hyclate antibiotic by using *P. lilacinum* strain PlHN17 and *T. asperellum* isolate Tullur.

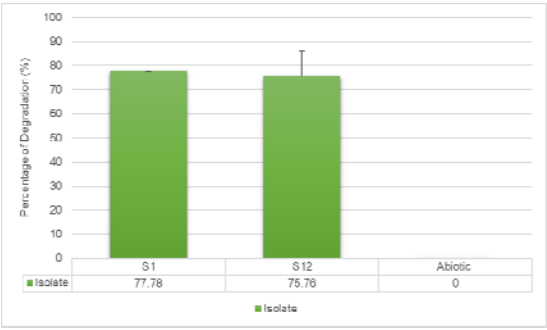

Figure 5. Percentage of degradation of doxycycline hyclate by *Purpureocillium lilacinum* strain PlHN17 (S1) and *Trichoderma asperellum* isolate Tullur (S12) in triplicates after 96 hours of incubation.

4. Conclusions

The isolated fungal species were identified morphologically and molecularly as *P. lilacinum* strain PlHN17 and *T. asperellum* isolate Tullur. *P. lilacinum* showed the highest degradation ability (77.78 %) to degrade doxycycline hyclate antibiotic. Hence, the isolated *P. lilacinum* showed a potential bioremediation tool to degrade doxycycline hyclate antibiotic.

Acknowledgements

This work is supported by Fundamental Research Grant Scheme (FRGS), 600-IRMI FRGS 5/3 (197/2019).

References

C˘vanc˘arová M, Moeder M, Filipová A and Cajthaml T. (2015). Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi--metabolites, enzymes and residual antibacterial activity. *Chemosphere* **136**: 311–320.

Carvalho T C de, Polizeli A M, Turatti I C C, Severiano M E, Carvalho C de, Ambrósio S R *et al.* (2010). Screening of filamentous fungi to identify biocatalysts for lupeol biotransformation. *Molecules* **15(9)**: 6140–6151.

Chopra I and Roberts M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **65(2)**: 232–260.

de Barros J O, Kogawa A C and Salgado H R N. (2018). Short-term stability study of doxycycline tablets by high performance liquid chromatography, spectrophotometry in the ultraviolet region and turbidimetry. *J. Pharmcol. Clin. Trials* **1(1)**: 43–49.

de Sequeira D C M, Menezes R C, Oliveira M M E, Antas P R Z, De Luca P M, Oliveira-Ferreira J and Borba C M. (2017). Experimental hyalohyphomycosis by *Purpureocillium lilacinum:*

Outcome of the infection in C57BL/6 murine models. *Front. Microbiol.* **8**: 1617.

Eliopoulos G M, Eliopoulos G M and Roberts M C. (2003). Tetracycline therapy: Update. *Clin. Infect. Dis.* **36(4)**: 462–467.

Javid A, Mesdaghinia A, Nasseri S, Mahvi A H, Alimohammadi M and Gharibi H. (2016). Assessment of tetracycline contamination in surface and groundwater resources proximal to animal farming houses in Tehran, Iran. *J. Environ. Health Sci.* **14(4)**.

Kim J Y, Yeo S H, Baek S Y and Choi H S. (2011). Molecular and morphological identification of fungal species isolated from bealmijang meju. *J. Microbiol. Biotechnol*. **21(12)**: 1270–1279.

Kogawa A C and Salgado H R. (2013). Quantification of doxycycline hyclate in tablets by HPLC-UV method. *J. Chromatogr. Sci.* **51(10)**: 919-925.

Melgar G Z, Assis F V S de, Rocha L C da, Fanti S C, Sette L D and Porto A L M. (2013). Growth curves of filamentous fungi for utilization in biocatalytic reduction of cyclohexanones. *Glob. J. Sci. Front. Res* **13(5)**.

Mohammad N S, Safian M F, Zainal Ariffin S H and Zainal Ariffin Z. (2018). Biotransformation of nitrofurans antibiotics by *Aspergillus* species -residual antibacterial activity. *Malays. J. Biochem. Mol. Biol.* **2**: 28–33.

Moreno-Gavíra A, Huertas V, Diánez F, Sánchez-Montesinos B and Santos M. (2020). *Paecilomyces* and its importance in the biological control of agricultural pests and diseases. *Plants* **9**.

Muthulakshmi, C., Gomathi, D., Kumar, D. G., Ravikumar, G., Kalaiselvi, M., & Uma, C. (2011). Production, Purification and Characterization of Protease by *Aspergillus flavu*s under Solid State Fermentation. *Jordan J Biol Sci*, ***4*(3)**.

Pena A, Albert-Garcia J R, Silva L J G, Lino C M and Calatayud J M. (2010). Photo-induced fluorescence of magnesium derivatives of tetracycline antibiotics in wastewater samples. *J. Hazard. Mater*. **179(1)**: 409–414.

Samuels G J, Lieckfeldt E and Nirenberg H I. (1999). *Trichoderma asperellum*, a New species with warted conidia, and redescription of *T. viride. Sydowia* **51(1)**: 71–88.

Saranya N, Suganya E, Narayanasamy S, Sivaprakasam S, Sivasubramanian V, Pandian S and Selvaraj R. (2020). 3-level Box–Behnkenoptimization of hexavalent chromium reduction by chromate resistant *Trichoderma asperellum* cells from simulated and industrial effluent. *Environ. Technol. Innov.*, **19**, 101024.

Sharma A, Sharma S, Mittal A. *et al*. (2016). Evidence for the involvement of nematocidal toxins of *Purpureocillium lilacinum* 6029 cultured on Karanja deoiled cake liquid medium. *World J Microbiol Biotechnol* **32**: 82.

Shanmugam S, Krishnaswamy S, Chandrababu R, Veerabagu U, Pugazhendhi A and Mathimani T. (2020). Optimal immobilization of *Trichoderma asperellum* laccase on polymer coated Fe_3O_4@SiO_2 nanoparticles for enhanced biohydrogen production from delignified lignocellulosic biomass. *Fuel* **273**: 117777.

Suda T, Hata T, Kawai S, Okamura H and Nishida T. (2012). Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole. *Bioresour. Technol.* **103(1)**: 498–501.

Xie J, Li S, Mo C, Xiao X, Peng D, Wang G and Xiao Y. (2016). Genome and transcriptome sequences reveal the specific parasitism of the nematophagous *Purpureocillium lilacinum* 36-1. *Front. Microbiol.* **7**:1084

Zafra G, Moreno-Montaño A, Absalón Á E and Cortés-Espinosa D V. (2015). Degradation of polycyclic aromatic hydrocarbons in soil by a tolerant strain of *Trichoderma asperellum*. *Environ. Sci. Pollut.* Res. **22(2)**: 1034–1042.

Zikmundová M, Drandarov K, Bigler L, Hesse M and Werner C. (2002). Biotransformation of 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one by endophytic fungi isolated from *Aphelandra tetragona*. *Appl. Environ. Microbiol.* **68(10)**: 4863–4870.