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# Detection and characterization of cadmium tolerant fungi from Kelantan river,Malaysia

**Nur Aisyah Zin[1*], Noor Afiza Badaluddin[1], Noraniza Awang Jaya[1], Mohammad Hailmi Sajili[1], Saiful Iskandar Khalit[1]and Muhamad Nazri Lokman[2]**

[1]School of Agricultural Sciences and Biotechnology, Faculty of Bio resources and Food Industry, Universiti Sultan Zainal Abidin, Malaysia, Besut Campus, 22200, Besut, Terengganu, **Malaysia.**
[2]Phytogold Sdn. Bhd.,No. 24, Jln P11C/7, Presint 11, 62300, Putrajaya, **Malaysia.**

***Correspondence:** nuraisyahzin95@gmail.com **Accepted: 26** June 2019 **Published online:** 15 Aug 2019

Nowadays, the increasing environmental pollution caused by toxic concentration of cadmium is a critical issue as it will affect the living organisms surrounding it. Biological approach such as using fungi as bioabsorption agent would be the best way in controlling the toxic concentration of cadmium. Thus, this present study aimed to identify and characterize the selected tolerant cadmium fungi from Kelantan river. Here, the detection of cadmium absorption level in the fungi were analysed by using atomic absorption spectroscopy(AAS) to determine whether it is potential as a bioabsorption agent or not. This study also detect the antagonistic characteristic of selected cadmium tolerant fungi towards some plant pathogenic fungi using dual culture assay. *Trichoderma hamatum*, *Trichoderma harzianum* and *Trichoderma atroviride* have been selected as potential cadmium tolerant fungi among 17 isolated fungal species in this study. They were good bioabsorption agent based on the percentage of reduction level of cadmium concentration detected by AAS. The cadmium tolerant fungal also showed high antagonist activity against the pathogenic fungal. Thus, this findings could be potential as one of a eco-friendly treatment for environment and biopesticide.

**Keywords: antagonistic activity, bioabsorption agent, cadmium tolerant fungi, *Trichoderma* sp.**

## INTRODUCTION

The soil contamination with the cadmium is significantly increasing as the result of industrial, mining, agricultural and domestic activities. The environmental pollution by heavy metal has been widely studied via heavy metal in water and soil (Krishna et al., 2009; Audry et al., 2004). It poses a serious threat to living things health that use the water from polluted river without any proper treatment process. Excessive dietary accumulation of cadmium (Cd) and lead (Pb) in the human body can lead to serious systemic health issues (Oliver, 1997).

Bioremediation/ bioabsorption involve the detoxification of hazardous substances such as heavy metal instead of transferring them from one medium to another, by using heavy metal tolerant fungal. Fungal absorption of heavy metal is an eco-friendly treatment method (Rahman et al., 2009). This process is characterized as less disruptive and less expensive compared to the chemical method that help eliminating the toxic concentration of heavy metal from polluted area. The identification of heavy metal tolerant fungal species is very crucial to contribute to bioremediation process. This method involves fungi that already resistance towards certain type of heavy metal to treat the polluted environment. Various type of polluted environments such as soil and river are commonly have the presence of *Trichoderma* sp. and a number of them have the capability of cleaning the contaminated area

(Vankar & Bajpai, 2008). Mohsenzadeh and Shahrokhi (2014) reported that *Trichoderma* sp. can uptake 76.17 % of cadmium in the optimal condition.

Therefore, this study aimed to identify the specific species of *Trichoderma* strain that have higher tolerance effect towards the cadmium ions, to determine absorption of cadmium by the selected cadmium tolerant fungi as well as to detect antagonistic characteristic of selected cadmium tolerant fungi towards some plant pathogenic fungi. The selected tolerant fungi can be used to treat the heavy metal polluted areas such as river and dam as bioabsorption agent.

## MATERIALS AND METHODS

### Sampling.

The sampling sites for fungi were conducted in three different polluted areas at Kota Bharu, Kelantan. The stems and leaves samples from this polluted areas were collected. All the collected samples were taken into the laboratory for isolation procedure.

### Isolation of fungi.

Method that was used in isolation of fungi is direct plating technique. The samples of stems and leaves that collected were isolated separately according to the different sampling sites. The samples of stems and leaves were cut into small fragments. Then, the samples were washed with 95% (v/v) EtOH for 1 min following by 5% (v/v) Clorox for 30 s and rinse with distilled water for 1 min to remove contamination surface of the samples by bacteria or other organisms. After that, the sterile samples were placed onto the potato dextrose agar (PDA). PDA is a nutrient-rich medium that can induce a wide range of fungi. The samples were incubated at room temperature in the dark for at least 5 days to induce sporulation. After 5 to 7 days, the colonies that grew were isolated to obtain pure culture. The pure culture was stored in the chiller for long term storage and was used in another experiment which is screening and identification method (Badaluddin, 2010).

Screening for cadmium tolerant fungi. The screening for fungi which are tolerant to cadmium toxicity were carried out by grown the fungi on PDA containing different concentration of cadmium sulfate (CdSO4) in the range of 0 to 1000 µM. All the fungi that have been isolated were placed on the agar. The agar plates were incubated in the dark for 7 days. The fungi that tolerates the highest concentration of cadmium were selected for identification.

### Fungal preparation.

Three unidentified selected cadmium tolerant fungi that showed highest tolerance towards toxic cadmium concentration were identified in this study by molecular method. Besides, six species of test pathogenic fungi were used in dual culture assay. Fungal cultivation for fungal identification and dual culture assay were done by using PDA and incubated at 30°C for optimum temperature growth. Preparation fungal sample for detection of cadmium absorption in fungal using atomic absorption spectroscopy (AAS) were prepared in potato dextrose broth (PDB) with medium shaking condition. Selected cadmium fungi used in this study were stored in agar slant for further purposes.

Extraction of genomic DNA. Molecular identification responsible to identify any living thing by using molecular probes that bind only to the DNA of targeted organism. The following preparations of mycelia were done according to modified method by Da silva et al., (2013). In preparation for DNA extraction, the selected cadmium tolerant fungi (KS3, KS4 and KS6) were grown first on PDA plates for 7 days at 30°C in the incubator. Approximately 100 mg of fresh mycelia was scraped from plates, placed in a 1.5-ml microfuge tube, macerated thoroughly with a micro-pestle, and allowed to stand for 6 to 10 min. The fresh mycelia was added with 200 µl of cell lysis solution and centrifuged for 1 min. After that, 600 µl of nuclei lysis solution was added followed by 1-3 sec of vortex and incubation at 65°C for 15 min. Three microliters of RNAse solution was added to the cell lysate and mixed the sample by inverting the tube 2-5 times. The mixture was then incubated at 37°C for 15 min and the sample was cooled to room temperature for 15 min before proceeding. Two hundred microliters of protein precipitation solution was added and vigorously vortex the microcentrifuge tube at high speed for 20 sec. Then, it was centrifuged at 13,000-16,000 rpm for 3 min. Tight pellet was formed due to precipitated protein. Next, the supernatant was transferred into a new microcentrifuge tube containing 600 µl of room temperature isopropanol. The solution was gently mixed by inversion until thread-like strands of DNA form a visible mass, followed by centrifugation at 13,000-16 ,000 rpm for 1 min at room temperature. The supernatant was discarded and the remaining pellet was washed with 600 µl of 70% ethanol and

then centrifuged. The ethanol carefully discarded, followed by air dry in incubator. Lastly, dissolved the DNA with 100 µl of DNA rehydration solution and rehydrate the DNA by incubating it for 65°C for 1 h. The DNA was stored at -20°C. The quality measurement of DNA had been done by mixing 5 µl of DNA with 1 µl loading dye. It then loaded onto 1.0% agarose gel. The sample was electrophoresed in 1x TBE buffer at 80 V for 120 min.

**Polymerase Chain Reaction (PCR).**

PCR was used to amplify the targeted DNA strands. Firstly, 50 µl of PCR reaction mixture was mixed in PCR tube. Then, it was inserted in Eppendorf Mastercycler Gradient. The PCR reaction mixture composed of 3.0 µM MgCl2, 1x PCR buffer (1 mM Tris-HCL and 5 mM KCl), ~100 ng of template DNA, 200 µM dNTPs, 1 U Taq Polymerase and 100 pmoles of each reversed and forward primer pairs. The primer pairs that used were LROR : 5' – ACCCGCTGAACTTAAGC – 3' and reverse primer ( LR7: 5' TACTACCACCAACATCT 3' ) . The conditions for PCR amplication that used was as follow: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec ,annealing temperature at 50°C for 1 min, extension at 74°C for 1 min 30 sec and final extension at 72°C for 3 min. PCR was ran for 34 cycles to reach sufficient amplification. The PCR products were separated by 2% agarose gel and stained with ethidium bromide to be visualized. The single band image with the predicted size without nonspecific bands or smear indicated that the PCR amplification process was a success.

**Purification of PCR product.**

The expected size of viewed bands was excised and gel purification conducted using the Wizard® PCR Preps DNA Purification System Protocol based on the manufacture's direction.

**DNA sequencing and blasting.**

The PCR product were sent for sequencing and the sequence adjustment of the sequences was done at http://www.ncbi.nlm.nih.gov/blast. The sequences recognized based on the molecular data on NCBI's integrated database.

Growth condition for cadmium absorption by fungus. The selected fungi was inoculated in 250 ml Erlenmeyer flasks containing 150 ml of potato dextrose broth (PDB) with 500 µM (0.384 mg/g) of cadmium. As control, no inoculation of selected tolerant fungi in another 250 ml. Erlenmeyer flasks containing 150 ml of PDB with at 150 rpm for 7 days.500 µM (0.384 mg/g) of cadmium. The cultures were incubated at 28°C in an rotary shaker

**Sample analysis.**

The mycelia were harvested by two stages of filtration. At first, the mycelia were filtered using a nitrocellulose filter with a pore size of 0.45 µm. Filtered PDB medium were used for determining total cadmium concentration. Then, the filtered PDB medium were filtered using sterile syringe filter to avoid blockage in any channel in AAS instrument. In order to achieved 0.3 mg/g of cadmium concentration for each sample, 1 µl of medium must be diluted with 49 µl of autoclaved water. The sample were sent to analysed its elements (cadmium) that quantified with standard cadmium solution using AAS.

Dual culture assay. The antagonistic activity of the selected cadmium fungi against six pathogenic fungi, *Fusarium* sp, *Fusarium oxysporum, Rhizoctonia solani, Colletotrichum truncatum, Corynespora cassiicola* and *Colletotrichum capsici* were studied by dual culture test by following the method of Rahman et al., (2009). Plate of PDA was inoculated with 7 mm disc from five-day-old cultures of selected fungi at the edge of the plate. Another agar disc of the same size of pathogenic fungi also was placed at the edge but on the opposite end of the same petri dish. As a control, pathogenic fungi was placed in the same position on another fresh PDA plate. All pairings were carried out in thriplicate and incubated at 30℃. Antagonistic activity was tested for 5 days after incubation by measuring the radius of the pathogenic fungi colony in the direction of the antagonist colony (R2) and the radius of the pathogenic fungi colony in the control plate (R1). The two readings were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson (2015)

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

**RESULTS**

**Isolation and screening of cadmium tolerant fungi**

A total of 17 fungal species have been successfully isolated from polluted area in Kelantan, Malaysia. They were coded by samples site and source of samples either stem or leaf. The coded name which are KL stands for

Kelantan Leaf and KS stands for Kelantan Stem. Eight fungal species successfully isolated from stem and nine fungal species isolated from leaf. All the fungal species then selected for further experiment which is screened with different concentration of cadmium. The result showed in Figure 1 that three fungi species can tolerated to highest cadmium concentration (1000 µM) were KS3, KS4 and KS6.

**Molecular identification of cadmium tolerant fungi**

Three selected cadmium tolerant fungi were identified by molecular identification process. Extraction of three fungus species labelled as KS3, KS4 and KS6 were obtained in high quality of DNA. All isolates were confirmed as *Trichoderma* sp. based on morphological and cultural characteristics. The quality of DNA extracts were measured by agarose gel electrophoresis. Figure 2 (A) shows the agarose gel electrophoresis of DNA of the putative fungal. PCR amplification used the DNA as templates for further analysis. The amplicons of approximately 1.5- kb as shown in Figure 2 (B) were amplified using the LROR forward and LR7 reverse primers by using 100bp DNA ladder in 2% agarose gel for electrophoresis process. DNA sequencing was then carried out for further confirmation of the identity of the selected cadmium tolerant fungi. They were identified as *T. hamatum*, *T. harzianum* and *T. atroviride* as shown in Table 1. Based on Table 1, the sequencing result of the PCR product from KS3 sample showed 99% homology with the large subunit of ribosomal RNA gene of *T. hamatum*, from KS4 sample showed 98% homology with 28S ribosomal RNA gene of *T. harzianum* and from KS6 sample showed 98% homology with 28S ribosomal RNA gene of *T. atroviride*.

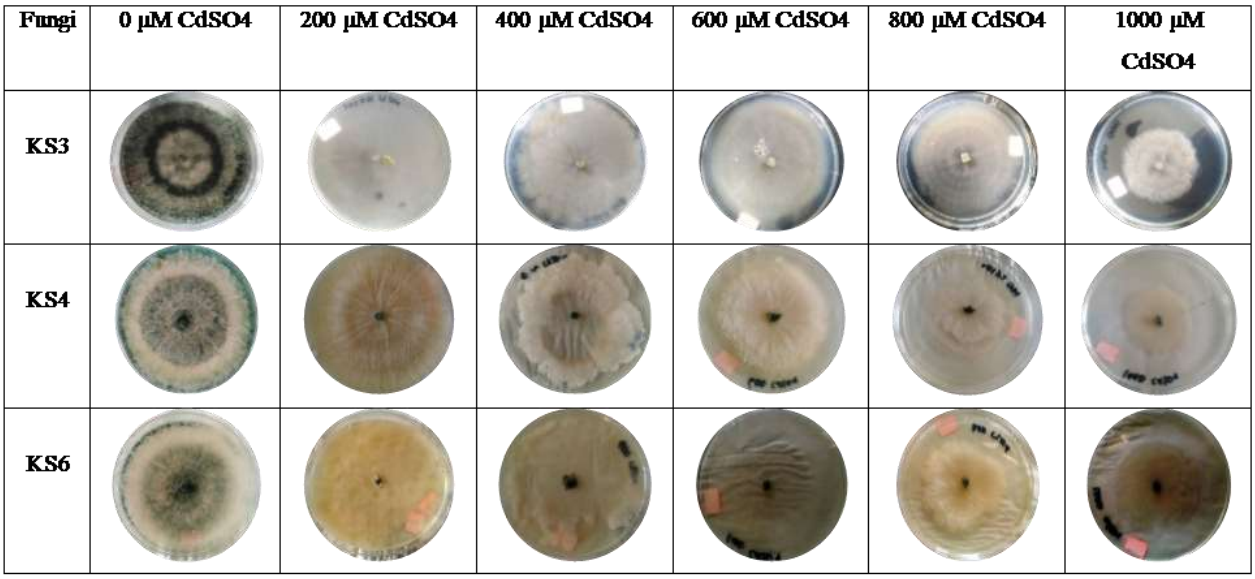


**Figure 1; Screening of fungi with cadmium tolerant property on PDA.**

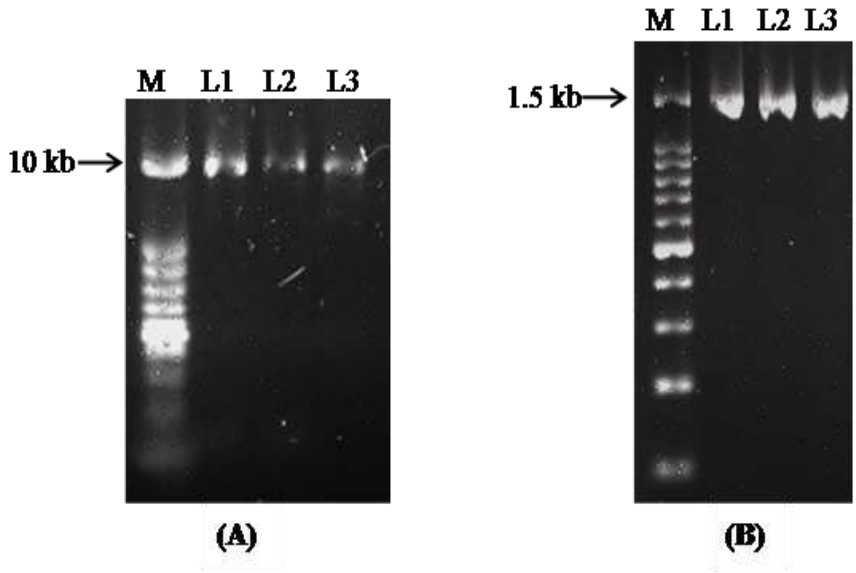


**Figure 2; (A) The bands of molecular mass DNA isolated from fungal sample on 1% agarose gel. M, 1kb DNA ladder. L1, the DNA extraction from KS3 sample. L2, the DNA extraction from KS4 sample. L3, the DNA extraction from KS6 sample. (B) Amplification of ~1.5 kb fragment of fungal. M, 100 bp DNA ladder. L1, PCR product of KS3 sample. L2, PCR product of KS4 sample. L3, PCR product of KS6 sample.**

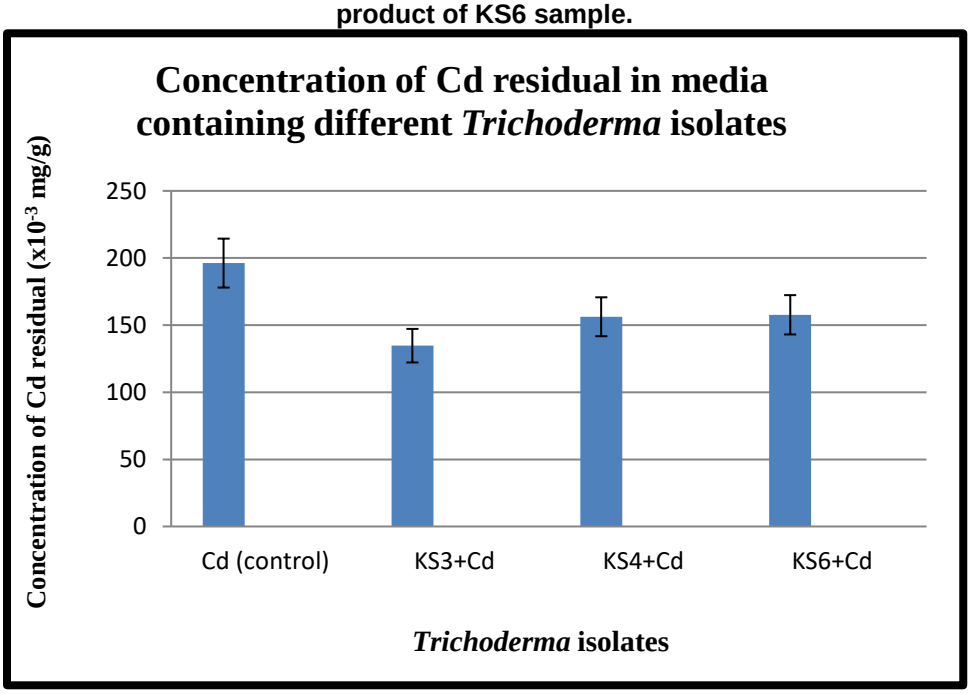


**Figure 3; The graph above depicts the concentration of cadmium (Cd) residual in potato dextrose broth (PDB) media containing cadmium sulphate (CdSO4) after 7 days.. Treatment; Cd (control) = PDB media only contain CdSO4, Cd+KS4= PDB media contain CdSO4 with inoculation of *T. hamatum*, Cd+KS4= PDB media contain CdSO4 with inoculation of *T. harzianum*, PDB media contain CdSO4 with inoculation of *T. atroviride.***

**Detection of cadmium absorption by selected cadmium tolerant fungi**

Based on the graph shown in Figure 3, the culture free control showed some decreased of cadmium concentration from 0.384 mg/g to 0.196 mg/g due to evaporated cadmium during high temperature. Nielsen (2010) stated that atomic absorption process in AAS use high temperature which is in between 1700°C and 3150°C. Cadmium eventually vaporized at 767°C (Balaram et al., 2012). KS3 showed the highest absorption of cadmium, evident from reduced initial concentration by 31.63% (0.196 mg/g to 0.134 mg/g). KS4 and KS6 reduced initial concentration by 20.40% and 19.89% (0.196 mg/g to 0.156 mg/g and 0.157 mg/g), respectively.

**Table 1: DNA sequencing result**

| Fungi sample | Accession | Description | Maximum Identity |
|---|---|---|---|
| KS3 | HM48886.1 | *Trichoderma hamatum* | 99% |
| KS4 | KC809921.1 | *Trichoderma harzianum* | 98% |
| KS6 | EF591763.1 | *Trichoderma atroviride* | 98% |

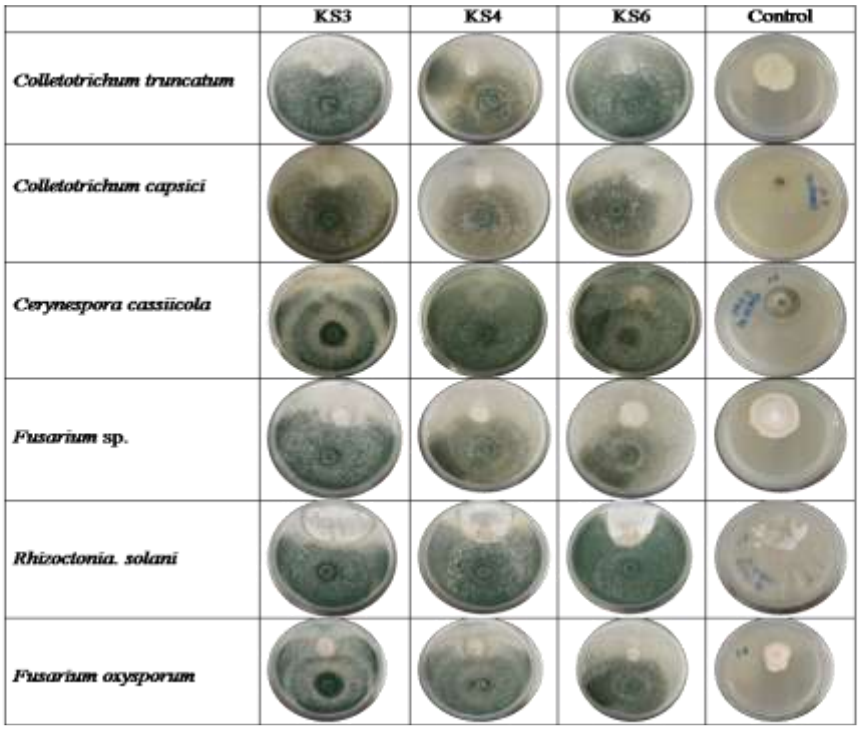


**Figure 4; Upper view of inhibition of pathogenic fungi by *Trichoderma* sp. isolate**

**Table 2 ; PIRG of selected cadmium tolerant fungi toward plant pathogenic fungi.**

| | Percentage Inhibition of Radial Growth(%) | | | | | |
|---|---|---|---|---|---|---|
| | *Corynespora cassiicola* | *Fusarium* sp | *Collectrichum truncatum* | *Fusarium oxysporum* | *Colletotrichum capsici* | *Rhizoctonia solani* |
| Control | 100 | 100 | 100 | 100 | 100 | 100 |
| KS3 | 70.31 | 63.64 | 60.31 | 48.78 | 60.26 | 83.48 |
| KS4 | 70.31 | 68.83 | 66.67 | 56.09 | 66.67 | 85.71 |
| KS6 | 67.19 | 62.33 | 63.49 | 48.78 | 65.38 | 83.04 |

**Dual Culture Assay**

Dual culture assay had been carried out to detect antagonistic characteristic of selected cadmium tolerant fungi towards some plant pathogenic fungi. Plant pathogenic that involved were *Colletotrichum truncatum, Colletotrichum capsici, Corynespora cassiicola, Fusarium sp., Rhizoctonia. solani* and *Fusarium oxysporum.* The inhibition radial growth of pathogenic fungi can be seen from the upper view shown in Figure 4. The experimental results showed that the three isolates of *Trichoderma* sp. tested were able to inhibit all the plant pathogenic fungi growth (between 48.78% and 85.71%) after 5 days, which showed significantly different with control ( p value < 0.05 ). The PIRG of selected cadmium tolerant fungi towards plant pathogenic fungus as shown in Table 2.

**DISCUSSION**

**Molecular identification process of cadmium tolerant fungi**

Methods for the extraction of DNA from filamentous fungi are frequently laborious and time consuming because most of the available protocols include maceration in liquid nitrogen after the mycelium has been grown in a liquid culture. Subsequently, the modified method by Da Silva et al., (2013) as described previously in extraction of genomic DNA method enabled in obtaining pure DNA from the sample in this study. In addition, the absence of maceration also reduces sample handling, minimizing the risks of contamination, a particularly important factor in work involving PCR.

**Cadmium absorption capability of selected cadmium tolerant fungi**

The result obtained can be strengthened further based on few studies that had been proven the ability of *Trichoderma* sp. to absorb cadmium. According to one of bibliographical studies, there was a report about cadmium absorption ability of *Trichoderma* species through certain metabolism called polyphosphate metabolism proven by *T. harzianum* species (De Freitas Lima et al. 2011). Besides, the first study on *T. asperellum* that can uptake cadmium in optimal condition was reported by Mohsenzadeh and Shahrokhi (2014). The most advantage of this finding is non-pathogenic properties of the studied fungi can be excellent bioabsorption agent in cadmium polluted area.

**Mechanism of *Trichoderma* sp. as biological control agent**

It had been proven scientifically in many studies on *Trichoderma* sp. ability to control plant pathogen (Avis et al., 2008). This antagonistic activities due to various mechanism involved such as enzyme biosynthesis that have antimicrobial properties and mycoparasitism. Elad et al., (1982) described in detail the mycoparasitism of *R. solani* hyphae by *T. hamatum* including coiling around pathogen hyphae, penetration, formation of appressorium like structure and resulting to degradation of the host cell wall.

Besides, enzyme biosynthesis by *Trichoderma* sp. have antimicrobial properties such as chitinases. Chitinases produced by the *Trichoderma* sp. are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity. Lorito et al., (1998) reported that transformants of tobacco and potato containing gene sequence encoding endochititinase from *T. harzianum* as in this study labelled as KS4 had successfully produce high level and broad spectrum of resistance against a number of plant pathogens.

Many researchers had proven that *T. atroviride* using the enzyme biosynthesis as a mechanism in the biocontrol process. Innocenti et al., (2003) reported that the *T. atroviride* show highest levels of glucosidase production which responsible to cell wall degrading enzymatic activities in pathogenic fungi. Recently, Bolar et al. (2000) demonstrated transgenic apple plants that had been transformed genes encoding chitinases from *T. atroviride* enhance their resistance

towards apple scab.

## CONCLUSION

Based on the above finding, the selected cadmium tolerant fungi (KS3,KS4,KS6) were identified as *T. hamatum, T. harzianum* and *T atroviride* via molecular method. They were good bioabsorption agents and have antagonistic characteristic towards selected plant pathogenic fungi.

## CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

## ACKNOWLEGEMENT

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## AUTHOR CONTRIBUTIONS

NAB devised the project, the main conceptual ideas and proof outline. NAZ contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript**,** with help from NAJ. MHS, SIK, and MNL helped supervise the project.



## REFERENCES

Audry, S., Schäfer, J., Blanc, G., & Jouanneau, J. M. (2004). Fifty-year sedimentary record of heavy metal pollution (Cd, Zn, Cu, Pb) in the Lot River reservoirs (France). Environmental Pollution, 132(3), 413-426.

Avis, T. J., Gravel, V., Antoun, H., & Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil Biology and Biochemistry, 40(7), 1733-1740.

Badaluddin, N.A. (2010) Isolation and Identification of Phytochelatin-Producing Fungi Associated with Mangrove. Universiti Malaysia Terengganu, Malaysia.( page 12)

Balaram S.,Nimai C. N.,Asutosh S. & Prafulla K. P. (2012). Inorganic Chemistry. New Delhi,India.( page 802 )

Bolar, J. P., Norelli, J. L., Wong, K.-W., Hayes, C. K., Harman, G. E., and Aldwinckle, H. S. 2000. Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology 90:72-77.

Da Silva, W. L., and Clark, C. A. 2013. Infection of sweetpotato by *Fusarium solani* and *Macrophomina phaseolina* prior to harvest. Plant Dis. 97:1636-1644.

De Freitas Lima, A., Ferreira de Moura, G., Barbosa de Lima, M. A., Mendes de Souza, P., Alves da Silva, C. A., de Campos Takaki, G. M., & Do Nascimento, A. E. (2011). Role of the morphology and polyphosphate in *Trichoderma harzianum* related to cadmium removal. *Molecules*, *16*(3), 2486-2500.

Elad, Y., Chet, I., & Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology, 28(7), 719-725.

Innocenti, G., Roberti, R., Montanari, M., & Zakrisson, E. (2003). Efficacy of microorganisms antagonistic to *Rhizoctonia cerealis* and their cell wall degrading enzymatic activities. Mycological research, 107(4), 421-427.

Krishna, A. K., Satyanarayanan, M., & Govil, P. K. (2009). Assessment of heavy metal pollution in water using multivariate statistical techniques in an industrial area: a case study from Patancheru, Medak District, Andhra Pradesh, India. Journal of hazardous materials, 167(1), 366-373.

Lorito, M., Woo, S. L., Fernandez, I. G., Collucci, G., Harman, G. E., Pintor-Toros, J. A., Filippone, E., Muccifora, S., Lawrence, C. B., Zoina, A., Tuzun, S., and Scala, F. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA 95:7860- 7865,

Mohsenzadeh, F., & Shahrokhi, F. (2014). Biological removing of Cadmium from contaminated media by fungal biomass of *Trichoderma* species. Journal of Environmental Health Science and Engineering, 12(1), 102.

Oliver, M. A. (1997). Soil and human health: a review. European Journal of Soil Science,

48(4), 573-592.
Rahman, M. A., Begum, M. F., & Alam, M. F. (2009). Screening of *Trichoderma* isolates as a biological control agent against *Ceratocystis paradoxa* causing pineapple disease of sugarcane. *Mycobiology*, *37*(4), 277-285.
Skidmore, A. M., & Dickinson, C. H. (1976). Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. Transactions of the British Mycological Society, 66(1), 57-64.
Nielsen, S. S. (Ed.). (2010). *Food analysis* (page 425). New York: Springer.
Vankar, P. S., & Bajpai, D. (2008). Phyto-remediation of chrome-VI of tannery effluent by *Trichoderma* species. Desalination, 222(1-3), 255-262.