Effects of Derivatization on the Metabolite Profiling of the Cadmium-Tolerant Mangrove Fungus *Trichoderma atroviride* using GC-MS Analysis

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Abstract

The mangrove fungus *Trichoderma atroviride* was found to be tolerant to the heavy metal cadmium and it is of high interest to profile its metabolites to gain insight into its response to cadmium toxicity. This study aimed to investigate the effect of derivatization agents on the number and types of metabolites present in the cadmium-tolerant *T. atroviride*, detected using GC-MS analysis. The intracellular and extracellular metabolites of *T. atroviride* treated with cadmium for ten days were derivatized using silylation and alkylation reactions. The results showed that a higher number of metabolites were identified when the three different derivatization agents were used: BSTFA, TBDMSTFA, and MCF. More types of metabolites were identified by silylation, making it suitable for non-targeted metabolites profiling study. Silylation is efficient for the analysis of sugars and their derivatives while alkylation is suitable for a targeted study involving amino acids and organic acids. Statistical analysis for the data set of identified metabolites was performed using Metaboanalyst 3.0 followed by visualization using Partial Least Square-Discriminant Analysis. The plots showed clear separations of metabolites between the different types of derivatization agents and between control and cadmium-treated samples. A more comprehensive metabolite profile of *T. atroviride* obtained using different derivatization agents in this study, followed by distinct metabolites detected between control and treated samples, will provide good baseline information for future investigations including the pathways and biomarkers responsible for the fungal tolerance to cadmium toxicity.

Keywords: alkylation, silylation, metabolomics, heavy metal, *Trichoderma*

Introduction

Heavy metal cadmium (Cd) is one of the pollutants in the environment with high toxicity for living organisms even at low concentrations (Suhani, Sahab, & Srivastava, 2021). In comparison to chemical remediation, bioremediation or removal of metal ions by microorganisms has attracted wide attention because of its low cost, easy operation, and high removal rate (Zhang, Ren, Zhong, & Wu, 2020; Meng, Peng, Pratush, Huang, & Hu, 2021). Among the microorganisms, fungi are good bioremediation agents owing to their robust morphology and diverse metabolic capacity (Deshmukh, Khardenavis, & Purohit, 2016). For example, *Trichoderma* spp. are among the most frequently found soil-borne fungi in different ecosystems with bioremediation potentials for various heavy metal pollutants including cadmium, lead, copper, zinc, chromium, and nickel (Yaghoubian, Siadat, Moradi Telavat, Pirdashti, & Yaghoubian, 2019).

Mangrove fungi have high potential as bioremediation agents as they are known to possess a high-stress tolerant ability which helps in maintaining the organometallic integrity among different substrata of the ecosystem (Chandra & Enespa, 2019). We have isolated a fungus from mangrove of Terengganu, Malaysia, identified as *Trichoderma atroviride* which showed high resistance towards cadmium toxicity (Jamali et al., 2020). To understand the mechanism of the fungal tolerance towards cadmium, profiling the metabolites produced upon exposure to the heavy metal is the first crucial step as different fungi react differently to metal stress. According to Robinson, Isikhuemhen, & Anike (2021), several methods of resistance are developed by fungi to counter metal toxicity, such as the alteration of the target protein to inhibit substrate

binding, cellular antimicrobial efflux, antimicrobial inactivation or degradation, restricted uptake to prevent cellular interference, overproduction of targeted proteins to prevent the complete inhibition of biochemicals, and compensation for loss of function directly related to the antimicrobial. The metabolomics approach has been widely applied in metabolites profiling to investigate the changes in metabolites upon exposure to heavy metals (Booth, Workentine, Weljie & Turner, 2011; Lankadurai, Nagato, & Simpson, 2013; Gajewska, Floryszak-Wieczorek, Sobieszczuk-Nowicka, Mattoo, & Arasimowicz-Jelonek, 2022). To the best of our knowledge, this is the first report on the metabolite profiling of a mangrove fungus from Terengganu, Malaysia upon exposure to cadmium toxicity.

In this study, Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to detect the metabolites for profiling. The fungus was grown in a medium with cadmium at its optimum tolerance condition, the mycelia were harvested, and both the intracellular and extracellular metabolites were extracted. Prior to GC-MS analysis, the metabolites were derivatized using silylation and alkylation methods, the two major derivatization procedures that have been described for GC-MS-based profiling of primary metabolites (Beale et al., 2018). In the silylation method, the active hydroxyl groups are converted to trimethylsilyl (TMS) ether or *tert*-butyldimethylsilyl (TBDMS) ether which reduces the polarity of the compound and reduces hydrogen bonding (Kühnel et al., 2007), resulting in more volatile and thermally stable metabolites for analysis (Orata, 2012; Schummer, Delhomme, Appenzeller, Wennig, & Millet, 2009). On the other hand, the methyl chloroformate (MCF) alkylation reaction converts primary and secondary amino groups into carbamate derivatives, whereas carboxylate groups are derivatized to form methyl esters, both of which can be analysed by GC-MS (Smart, Aggio, Houtte, Van, & Villas-bôas, 2010). Multivariate data analysis was also performed to visualise metabolite distribution in different derivatization methods.

This study aimed to investigate the effect of derivatization agents on the number and types of metabolites present in the cadmium-tolerant *T. atroviride*, detected using GC-MS analysis. It is crucial to identify all types of metabolites present in the fungus to obtain a comprehensive metabolome dataset. The findings will provide the important baseline data required for future studies including the pathways and biomarkers responsible, to gain insight into the response mechanism to cadmium toxicity by *T. atroviride*, the mangrove fungus of Terengganu, Malaysia.

Material and Methods

Cultivation of *T. atroviride* in Medium with Cadmium

The *T. atroviride* culture was previously isolated from the mangroves of Terengganu, Malaysia (Jamali et al., 2020). For spore isolation, *T. atroviride* was cultured on Potato Dextrose Agar (PDA) at 30 °C in dark conditions for 7 to 14 days. Spores were collected from the disk culture by scraping the fungus from the PDA into a 2 ml microcentrifuge tube containing 1 ml sterile mili-Q water. The suspension was then diluted to 4 ml in a 10 ml centrifuge tube and vortexed gently and briefly. The suspension was then transferred into the open end of a 5 ml syringe (plunger removed) with the tapered end of the syringe packed with sterile glass wool. The plunger was returned to the syringe and the spore suspension was eluted into a 15 ml centrifuge tube. Then, another 6 ml of sterile mili-Q water was passed over the glass wool and eluted into the same tube. The spores were collected by centrifugation at 5000 rpm and the supernatants were discarded. The spores were then diluted as appropriate and counted using a hemocytometer.

For cultivation, about 1×10^6 spores/ml were inoculated in 400 ml Potato Dextrose Broth (PDB) media added with four different concentrations of filter-sterilized $CdCl_2$ solution: 0, 0.75, 1.5 and 2.0 mM (Jamali et al., 2020). The cultures in triplicates were incubated further in an incubator shaker at 30 °C and 150 rpm for 14 days. For control, *T. atroviride* grown in PDB without the addition of $CdCl_2$ solution was used.

Determination of Fungal Biomass Upon Exposure to Cadmium

After the incubation period, the fungal growth was estimated as mycelial biomass (g/L of the culture medium). The fungal mycelia were harvested at every 48-hour interval, washed with 75% methanol, and separated from the liquid culture by filtration through a Whatman No. 1 filter paper. The mycelial pellet was repeatedly washed with a rinsing solution (50 % methanol) and dried at 70 °C for 12 h to determine the mycelial dry weight, using the following formula:

Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)

The average dry weight of the triplicates was used to plot the growth curve of *T. atroviride* (Yaghoubian, et al., 2019).

Fungal Materials for Metabolomics Analysis

Based on the optimum condition for cadmium tolerance identified in Section 2.2, spores of *T.*

atroviride were inoculated in 400 ml PDB added with filter-sterilized 0.75 mM $CdCl_2$ solution, and the culture was incubated further in an incubator shaker at 30 °C and 150 rpm in the dark for 10 days. For control, *T. atroviride* grown in PDB without the addition of $CdCl_2$ solution was used. Five biological replicates from each treatment were sampled where the fungal mycelia were harvested at day 10. For the mycelial harvest, the metabolism of the fungal mycelia was simultaneously slowed/stopped by adding 800 ml of Quenching Solution (75% methanol solution, -20 °C) into the flask culture. The mycelia were quickly harvested through vacuum filtration and transferred to a 5 ml centrifuge tube and frozen in liquid nitrogen. The fungal mycelia then were dried by lyophilization and stored at -80 °C prior to metabolite extraction (Gummer et al., 2012).

Extraction of Metabolites

For the extraction of intracellular metabolites, 18 mg of dried fungal mycelia for each biological replicate was transferred to a 15 ml centrifuge tube and 6 ml -20 °C methanol was added. The mixture was then sonicated for two min using a probe-type ultrasonic homogenizer and then mixed by the vortex. The supernatant was collected by centrifugation at 1000 rpm for 15 min and transferred into a fresh centrifuge tube. Two ml of -20 °C methanol was added to the remaining pellet and the mixture was homogenized for another two min. The supernatant again was collected by centrifugation at 1000 rpm for 15 min and was added to the previous supernatant. The pellet was discarded. The combined supernatant was mixed by vortex and dried by evaporation in a vacuum concentrator in preparation for metabolite analysis (Gummer et al., 2012; Madla, Miura, & Wariishi, 2012).

For the extracellular components, the filtrate collected during the harvest was vortexed and transferred to a fresh centrifuge tube and frozen in liquid nitrogen. The extracellular components then were dried in a centrifugal evaporator followed by lyophilisation (Gummer et al., 2012). Freeze-dried samples were stored at -80 °C until further use.

Derivatization of Samples

For silylation, two types of agents were used: N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka) and N-*tert*-Butyldimethylsilyl-N-methyltrifluoro-acetamide (TBDMSTFA, Acros Organic) according to protocols described by Gummer et al. (2012) and Azizan, Baharum, and Mohd Noor (2012). In silylation with BSTFA, 20 µl Methoxyamine HCl (20 mg/ml in pyridine, Sigma Aldrich) was added to a 2 mg dried sample and transferred into a glass insert in the 2 ml vial. Then, 20 µl BSTFA was added and vortexed before incubation at 60 °C for 60 min with shaking at 1250 rpm. The final mixture was concentrated using a nitrogen gas concentrator and redissolved with isooctane before it was placed in the GC-MS for analysis. In silylation with TBDMSTFA, 20 µl of Dimethylformamide (DMF, Sigma Aldrich) was added to the dried sample and quickly transferred into a glass insert in the 2 ml amber-sealed vial. Then, 20 µl of TBDMSTFA with 1% *tert*-Butyldimethylchlorosilane (TBDMCS, Sigma Aldrich) was added, and the mixture was incubated at 85 °C for 1 hr. The final mixture was concentrated using a nitrogen gas concentrator and redissolved with isooctane before it was placed in the GC-MS for analysis.

The alkylation method was carried out using Methyl Chloroformate (MCF, Sigma Aldrich) based on the protocol described by Smart et. al (2010) and Villas-bôas, Smart, Sivakumaran, and Lane (2011). The dried sample was redissolved in 200 µl of NaOH solution (1 M) and mixed with 167 µl of methanol and 34 µl of pyridine. Then, 20 µl of MCF was added and mixed vigorously using a vortex for 30 s. Another 20 µl MCF was added to the reaction mixture followed again by vigorous mixing for another 30 s. Approximately 400 µl of chloroform was added to the mixture followed by vigorous mixing for 10 s, the addition of 400 µl $NaHCO_3$ solution (50 mM), and mixing again for another 10 s. The upper layer was discarded, and the remaining solution was dried using sodium sulphate. The final mixture was placed in the GC-MS for analysis.

GC-MS Analysis

For each of the five replicates, three technical replicates were used for analysis. GC-MS analysis was performed using the Perkin Elmer TurboMass 600 Clarus spectrometer coupled to a quadrupole mass selective detector on electron ionization (EI) operated at 70 eV. A sample aliquot of approximately 1 ìl was injected into the 5MS capillary column coated with 5 % diphenyl 95 % dimethyl polysiloxane (ID 30 m × 0.25 mm × thickness 0.25 µm, Perkin Elmer, USA). The oven temperature was initially held at 70 °C and raised until 170 °C with a gradient of 10 °C/min; the temperature was then raised with a gradient of 30 °C/ min until 280 °C and held for three min. The helium flow was held constant at 1.1 mL/min and the injection was set at 1 µL. The injection was set under a split ratio of 20:1. The inlet temperature was 250 °C and the quadrupole temperature was set at 300 °C. The measurements were performed in scan mode of m/z 50-500.

Data Analysis and Validation

Detected peaks were identified using the 2008 National Institute of Standards and Technology (NIST) mass spectral database library (NIST) with a similarity cut-off of 80%. The selection of the NIST library as the spectrum library for matching and 80% similarity as a cutoff point is similar to what is used in other studies on volatile organic compound (VOC) identification (Kamatou, & Viljoen, 2017; Sola Martínez et al., 2020). Metabolomics Ion-based Data Extraction Algorithm (MET-IDEA) software was used in peak alignment, annotation, and integration. Each of the identified metabolites was then checked with the Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/), The Human Metabolome Database (HMDB, http://www.hmdb.ca/), and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). The values of peak area were used to represent the detected metabolites. Statistical analysis for the data set was performed using Metaboanalyst 3.0 (Xia & Wishart, 2016). The data matrix was then normalized to the internal standard and total sum of Total Ion Chromatography (TIC) followed by log transformation and subsequently by statistic validation using one-way Analysis of Variance (ANOVA) and compared using Fisher's Least Significant Difference (LSD) method with significant levels of $p<0.05$. Visualization of the clean and validated data was then analyzed using Partial Least Square-Discriminant Analysis (PLS-DA) in SIMCA-P+ version 14.0 (Umetrics AB, Ume, Sweden) for group classification and discrimination analysis.

Results and Discussion

Effect of Cadmium Toxicity on the Growth of *T. atroviride*

The growth pattern based on the mycelial mass of *T. atroviride* in PDB upon exposure to four different concentrations of cadmium as compared to control is shown in Figure 1. In the absence of cadmium, *T. atroviride* grew at a rapid rate where the fungus achieved its optimum growth on day 8 and started to decline towards day 14 of incubation. On the other hand, the concentration of cadmium in the culture medium affected the growth pattern of *T. atroviride*. In general, the growth of *T. atroviride* in the three concentrations of cadmium was slower than that of the control, particularly until day 4. In fact, in 1.5- and 2.0-mM cadmium, the biomass of *T. atroviride* was almost similar until day 14 of incubation. At a lower cadmium concentration of 0.75 mM, the fungus entered the exponential phase after day 4 and achieved the maximum biomass at day 10 before decreasing towards day 14 of incubation.

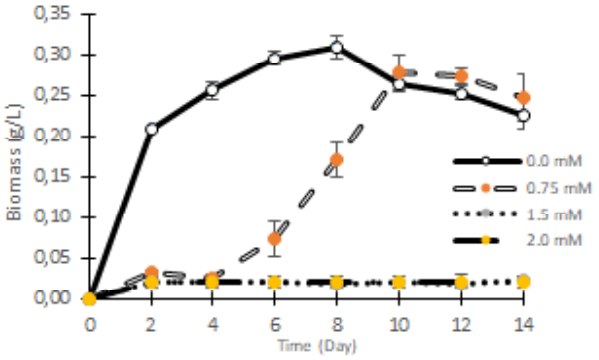

Figure 1. Biomass of *T. atroviride* exposed to different concentrations of cadmium for 14 days at 30 ^{0}C.

The result for the non-treated fungus in this study is in line with the report by Abdelghany and El-Sheikh (2016) who stated that normal fungal growth against time typically shows an S-shaped growth curve, where the log and exponential phases require three and six days, respectively, achieving optimum growth within six to eight days before reaching the stationary phase from day 8 onwards. Sinha, Singh, Rao, and Verma (2018) also reported almost similar days of optimum growth which is 7 days on PDA at 25 °C, upon performing comprehensive evaluation on the growth of *Trichoderma harzianum* and *T. viride* on different culture media at different temperatures and pH. As for the cadmium-treated fungus, the results suggested that although *T. atroviride* could tolerate cadmium toxicity in the medium, the growth rate was affected, as indicated by Jamali et al. (2020). Bano et al. (2018) reported that the growth of various species of *Aspergillus* spp. and *Sterigmatomyces halophillus* was observed to be inhibited by heavy metals like Cu, Cd, Mn, Pb, and Zn. Similarly, Graz, Pawlikowska-Pawlega, & Jarosz-Wilkolazka (20110 showed that Pb inhibited the growth of *Aspergillus biennis* at a concentration of 10-30 mM range, while Cd inhibited the growth of *Schizophyllum commune* at a concentration of 0.1-0.2 mM (Priyadarshini, Priyadarshini, Cousins, & Pradhan, 2021).

Studies have reported that heavy metal ions adversely affect the fungal cellular life process which leads to the inhibition of growth and death. According to de Oliveira and Tibbett (2018), a reduction in fungal growth by heavy metals can be caused by the inhibition of enzymes and disruption of mitochondrial membranes. Ayangbenro and Babaloa (2017) reported that high accumulation of cadmium in microbes induces protein and nucleic acid damage resulting in inhibition

of cell division and transcription, while Hg ions disrupt the microbial cell membrane, impair enzyme function, and denature proteins.

Based on the results, for the following study on metabolomics analysis of *T. atroviride* upon exposure to cadmium, the fungus was grown until day 10 in 0.75 mm $CdCl_2$ which represented the optimum tolerance to cadmium.

Effects of Different Derivatization on The Identification of Metabolite

Table 1 shows that a total of 116 metabolites were identified for the intracellular samples (Supplementary S1) while a total of 94 metabolites were identified for the extracellular samples (Supplementary S2). The total of 210 metabolites detected by the combination of all three derivatization agents would provide a more comprehensive metabolite profile of the mangrove fungus in response to cadmium toxicity. Compared to the use of a single derivatization agent, only 84, 66, and 60 metabolites were identified by BSTFA, TBDMSTFA, and MCF, respectively. Between the two silylation methods, BSTFA identified more metabolites compared to TBDMSTFA. On the effect of the derivatization agent on the types of metabolites identified, silylation identified all groups of metabolites (except for no amino acids identified by BSTFA intracellularly), while MCF did not identify any sugar metabolites and polyols. Furthermore, the results showed that BSTFA identified sugars the most in both intracellular and extracellular samples. On the other hand, TBDMSTFA identified a low number of sugars. Interestingly, TBDMSTFA detected the highest number of amino acids in the intracellular sample. As for alkylation, the method resulted in the identification of amino acids, organic acids, and fatty acids.

The finding that shows BSTFA identified more metabolites compared to TBDMSTFA is in line with the report by Abbiss, Rawlinson, Maker, and Trengove (2015), where upon the derivatization of rat urine samples, BSTFA showed a significantly greater TIC intensity compared to the MSTFA reagent. In addition, BSTFA has also long been the preferred silylation reagent compared to other commonly used silylation reagents, such as BSA, MSA, and MSTFA (Little, 1999). Better identification of metabolites by silylation method as compared to alkylation may be due to their comprehensive coverage of compound classes and their relative ease of use, and hence Trimethylsilylation (TMS) become the most commonly used reagents (Kanani & Klapa, 2007). Overall, trimethylsilylation offers the broadest access to small molecules under mild and universal reaction conditions (Kvitvang, Andreassen, Adam, Villas-Bôas, & Bruheim, 2011; Villas-Boas et al., 2011). The priority of small molecules for trimethylsilylation is generally in the order: Alcohols > Phenols > Carboxylic Acids > Primary Amines > Secondary Amines > Amides (Orata, 2012).

As for the results that showed BSTFA identified sugars the most while TBDMSTFA identified a low number of sugars, this may be because TBDMS derivatives are very bulky, increasing steric hindrance leading to incomplete derivatization of polyhydroxy metabolites such as sugars (Lai and Fiehn, 2018). Interestingly, the finding that indicated TBDMSTFA detected the highest number of amino acids in the intracellular sample is in line with the findings by Jiménez-Martín, Ruiz, Pérez-Palacios, Silva, and Antequera (2012) who used TBDMSTFA for the simultaneous analysis of 22 free amino acids in a variety of food sources by GC–MS and found that all of them were correctly detected and resolved, indicating the high capability of TBDMSTFA in detecting amino acids. This study also shows that alkylation identified amino acids, organic acids, and fatty acids, which is similar to the finding by Azizan et al. (2012) who stated that metabolites detected by MCF

Table 1. The number of *T. atroviride* metabolites detected by CG-MS analysis with $p < 0.05$ in intracellular and extracellular samples. The metabolites include both control and cadmium-treated *T. atroviride*

Metabolite Group	Intracellular Metabolites				Extracellular metabolites				
	BSTFA	TBDMSTFA	MCF	Total	BSTFA	TBDMSTFA	MCF	Total	Grand total
Amino acids	7	17	5	29	0	3	11	14	43
Fatty acids	6	9	8	23	3	3	4	10	33
Organic acids	8	10	6	24	2	10	8	20	44
Sugars	14	3	0	17	22	4	0	25	38
Polyols	3	1	0	4	2	0	0	3	7
Others	7	1	11	19	10	5	7	22	45
Total	45	41	30	116	39	25	30	94	210

in *Lactococcus lactis* grown under different culture conditions were mainly amino acids and organic acids, including metabolites from the citrate cycle and fatty acids. This is because the target compounds of chloroformate reagents are mostly the primary and secondary amines, amides, sulphonamides, thiols, phenols, carboxylic acids, and alcohols (Söderholm, Damm, & Kappe, 2010).

To support the advantage of using different derivatization agents to obtain more varieties of metabolites for profiling, we identified metabolites that were either detected by all three derivatization agents or any two of the agents (Figure 2). The Venn diagrams indicate that more metabolites that are different, were identified by each derivatization agent. For instance, out of 29 amino acids identified in the intracellular metabolites (Figure 2A), only 8 amino acids were found identified by either all three agents or any two of the agents. In addition, the intracellular sugars identified by BSTFA and TBDMSTFA are completely different, while 19 different extracellular sugars were identified by BSTFA, compared to one sugar identified by TBDMSTFA (Figure 2B). The more different metabolites were identified, the more comprehensive metabolite profiling of *T. atroviride* can be performed in the future cadmium-tolerance study.

In this study, three different types of derivatization agents were used for GC-MS analysis involving silylation reactions (BSTFA and TBDMSTFA) and alkylation reactions (MCF) to identify all possible metabolites in the cadmium-tolerant *T. atroviride*. This is because, for an untargeted metabolomics approach, in particular, comprehensive metabolome datasets can only be obtained using a combination of a variety of sample preparation protocols and multiple analytical instruments (Duportet, Aggio, Carneiro, & Villas-Boas, 2012; Pinu, Villas-Bôas, & Aggio, 2014). Furthermore, the chemical diversity and variable nature of metabolites from the various types of biological material that can be sampled in any one study are extensive, and therefore a single analytical technique is not sufficient to detect, identify and quantify all the possible metabolites that may be present (Wishart, 2016).

Evaluation of Different Derivatization Protocols using PLS-DA

In this study, Partial Least Square-Discriminant Analysis (PLS-DA) was used to visualize the grouping patterns and to detect the outliers in the data sets by comparing the variations derived from 5 replicates in each different derivatization protocol. PLS-DA models were usually explained by the squared correlation coefficient (R^2) and predictive ability (Q^2) values. The score plot for intracellular and extracellular samples illustrated a clear and reasonable clustering according to derivatization methods and sample groups (Figures 4 and 5). The group separation of the intracellular PLS-DA model achieved with a coefficient of determination (R^2) exhibited 60.2 %, 92.2 % for R^2X, and 98.9% for R^2Y, and a predictive score (Q^2(cum)) of 97.3%. While the extracellular PLS-DA model resulted in 42.4 % for R^2, 79.3 % for R^2X, and 96.7 % for R^2Y, and a predictive score (Q^2 (cum)) of 94 %. The model fitness and the predictive ability were considered good when the values were greater than 0.5 (close to 1).

In addition, there was a significant separation between the cadmium-treated group and the control group. In the intracellular samples, control metabolites for MCF were further separated from cadmium-treated metabolites in the lower quadrant of the PLS-DA plot,

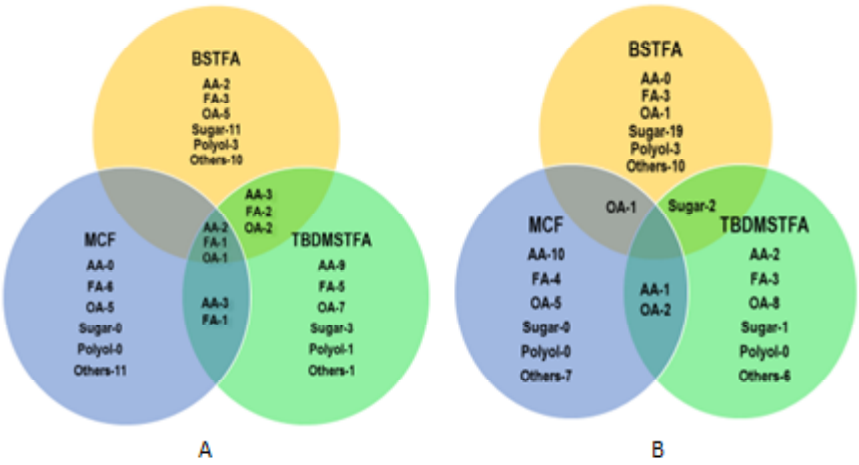

Figure 2. The number of overlapping metabolites with $p<0.05$ detected by CG-MS analysis in (A) intracellular samples and (B) extracellular samples. The metabolites include both samples for control and cadmium-treated *T. atroviride*. AA-Amino acids; FA-Fatty acids; OA-Organic Acids.

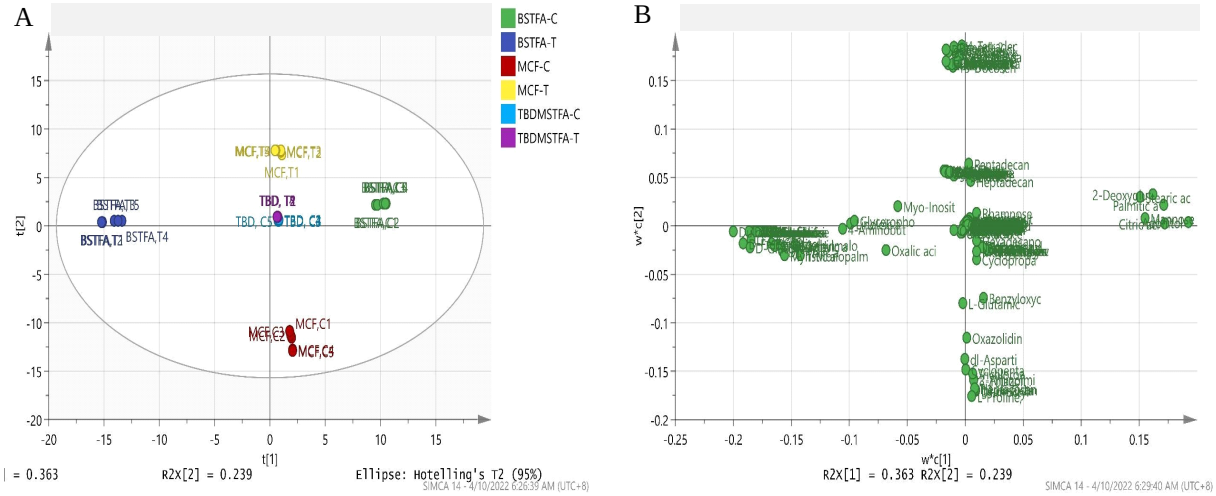

Figure 3: The PLS-DA score plot (A) and loading plot (B) of intracellular *T. atroviride* metabolites analyzed using GC-MS with different derivatization agents at day 10. The ellipse represents the 95% confidence region for Hotelling's T^2

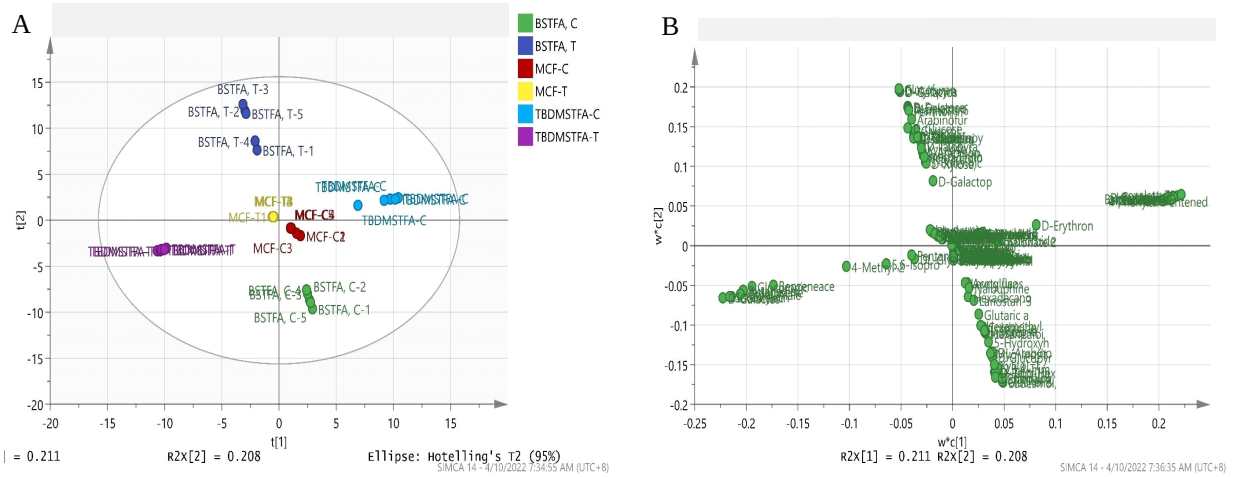

Figure 4: PLS-DA score plot (A) and loading plot (B) of extracellular *T. atroviride* metabolites analyzed using GC-MS with different derivatization agents at day 10. The ellipse represents the 95% confidence region for Hotelling's T^2.

while control metabolites for BSTFA were separated at the right quadrant from cadmium-treated metabolites. This indicates a strong variation in the metabolite concentration and composition of the PLS-DA plot. TBDMSTFA metabolites were closely clustered in the middle of the score plot, indicating less variation in the metabolite concentration and composition of this method. As for the extracellular sample, control metabolites for TBDMSTFA were located in the right quadrant far from cadmium-treated metabolites. BSTFA metabolites were also separated at the lower and upper quadrants of the PLS-DA plot. Both metabolites for the MCF group were located at the centre of the score plot but were still separated.

Results obtained from PLS-DA models indicating the significant differences between the three derivatization methods, together with the distinct boundaries observed between the cadmium-treated group and control group, indicating a conspicuous difference in their response to the cadmium stress, would provide reliable data for further study on the identification of the metabolites responsible for cadmium toxicity response by *T.atroviride*.

Conclusion

This study was carried out to investigate the effect of derivatization agents on the number and types of metabolites present in the cadmium-tolerant *T. atroviride*, detected using GC-MS analysis. The results showed that the combination of all three derivatization agents identified a higher number of metabolites present in the cadmium-tolerant *T. atroviride*, providing a more comprehensive metabolome dataset. BSTFA identified more metabolites than TBDMSTFA, while MCF is

suitable for a targeted study, such as the identification of amino acids and organic acids. Between the two derivatization methods, silylation detected a wider range of chemical groups compared to alkylation, making it a preferable derivatization method for metabolic profiling experiments. In addition, the three derivatization agents identified more metabolites distinct from each other with only a small number of overlapping metabolites identified between them. Furthermore, PLS-DA models showed clear discrimination between the three derivatization methods, as well as distinct separation between the cadmium-treated and control groups. The metabolite profiles obtained from the study can be used in further investigations to gain insight into the response mechanism to cadmium toxicity by the mangrove fungus *T. atroviride*. It is worth noting the limitation in using GC-MS analysis where the derivatization methods required to convert many metabolites to volatile derivatives involve multiple derivatization steps and underivatized compounds, which may result in GC-MS artifacts. Therefore, more studies are required before *T. atroviride* can be recommended as the potential bioremediation agent for heavy metal pollutants

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Supplementary Materials

Supplementary materials are available online at the Journal's website.

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