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Ability of endophytic fungi isolated from *Nepenthes ampullaria* to degrade polyurethane

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ABSTRACT

Aims: Waste electric and electronic equipment (WEEE) are among the fastest growing waste products worldwide and solutions to their remediation are urgently needed. Bioremediation is a green approach that is helpful to minimize environmental pollution associated with Electronic waste (E-waste). The present study aimed at exploring the potential of endophytic fungi from *Nepenthes ampullaria* for bioremediation purposes of the plastic component in E-waste, polyurethane (PUR) polymers.
Methodology and results: Endophytic fungal isolates were assessed for their ability to degrade PUR as well as their ability to utilise PUR as sole carbon source. Nine (9) out of 150 isolates demonstrated the ability to efficiently degrade polyurethane in solid medium and the top three (3) isolates were able to grow on PUR as the only carbon source. These three isolates were identified using ITS1 and ITS4 and found to be closely related to the genus *Pestalotiopsis*. The top two of the three isolates were then assessed for their esterase enzyme activity as well as changes in their proteome when grown with and without PUR. The highest enzymatic activity was found to be 1850.4 U/mL when tested using *p*-nitrophenol acetate as the substrate. Analyses of the 2-dimensional electrophoresis profile revealed changes in the abundance of proteins when treated with polyurethane.
Conclusion, significance and impact of study: This study is to our knowledge the first on endophytes isolated from *N. ampullaria* that can degrade PUR, and also their proteomes. Results obtained from this study can in the future help to reduce polyurethane wastes. Besides degrading PUR polymer, endophytic fungi produce potential valuable proteins that may find broad applications in bioremediation applications.

Keywords: Biodegradation, *Pestalotiopsis*, *Nepenthes*, Polyurethane, Proteome

INTRODUCTION

Waste electric and electronic equipment (WEEE) has been recognized by the European Union (EU) as one of the fastest growing waste streams in the region, with an estimation of up to 20 kg of WEEE per person being dumped every year (Darby and Obara, 2005); accounting for approximately 8% of the total solid waste in EU member countries (Wath *et al.*, 2010). It has been estimated that 20-50 million tons of E-waste are discarded annually with Asian countries accountable for around 12 million tons (Cobbing, 2008). The largest generator of E-waste in the world is the USA with a total accumulation of 3 million tons per year while China is the second largest, generating 2.3 million tons every year (Oliveira *et al.*, 2012).

Waste electric and electronic equipment is predominantly a mixture of metals and plastics. Plastics are lightweight and essential as an electric or thermal insulator. The portion of plastics in EEE has constantly increased from about 14% in 1980 to 18% in 1992, and 23% in 2005 (Buekens and Yang, 2014).

Resource depletion and litter problems have occurred due to the massive quantities of used PUR-containing equipment that are disposed in landfills and incinerators (Zheng *et al.*, 2005). Several studies have demonstrated that groundwater contamination can be caused by landfills accepting electronic devices or old landfills containing E-wastes (Yang, 1993; Schmidt, 2002). Pollutants have the potential to migrate within and around

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landfill sites through soils and groundwater (Kasassi *et al.*, 2008). The problem of littering and surface water pollution is intensified by the fact that plastics are not biodegradable in landfills (Rowe and Howard, 2002; Mukherjee *et al.*, 2011; Russell *et al.*, 2011). Hence, novel bioremediation approaches are and need to be developed for plastic degradation utilizing microorganisms.

Several reports revealed the use of fungi and bacteria to degrade PUR. Both PUR-degrading fungi (Bentham *et al.*, 1987; Barratt *et al.*, 2003; Sabev *et al.*, 2006) and bacteria (Kay *et al.*, 1991; Akutsu *et al.*, 1998; Howard *et al.*, 1999) have been isolated from soil, signifying that there are potential reservoirs of PUR-degrading organisms widespread in the environment. *Comamonas acidovorans* strain TB-35 was shown to be able to utilize PUR as a sole carbon and nitrogen source (Nakajima-Kambe *et al.*, 1995; Akutsu *et al.*, 1998). An automotive waste polyester PUR foam was also successfully degraded by microorganisms using *Pseudomonas chlororaphis* (Gautam *et al.*, 2007). A number of fungal species such as *Curvularia senegalensis, Fusarium solani, Aureobasidium pullulans* and *Cladosporium* sp., were isolated from soil and are capable of degrading ester-based PUR (Crabbe *et al.*, 1994).

A range of microbial species are known to be endophytic, colonizing inter and intracellular spaces of tissues of higher plants without causing apparent damage on the plants in which they live (Sharma *et al.*, 2016). Mutualistic interactions between endophytes and host plants may result in benefits for both partners (Kogel *et al.,* 2006). The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine (Porras-Alfaro and Bayman, 2011; Wong *et al.*, 2015). Endophytic fungi are of biotechnological interest and studies have reported that endophytic fungi can survive under high metal concentrations (Kapoor *et al.*, 1999; Yan and Viraraghavan, 2003; Onn *et al.*, 2016) and able to degrade PUR (Russell *et al.*, 2011).

About 500 species of vascular plants trap and digest animals in order to gain additional inorganic nutrients (Juniper *et al.*, 1989; Barthlott *et al.*, 2004). The retention of animals is performed by different mechanisms such as sticky adhesive traps, moveable snap traps, pitchers, suction bladders or eel traps (Lloyd, 1942). The trapped animal is dissolved by digestive enzymes produced either by the plant itself (carnivorous plants) or by mutualistic organisms (protocarnivorous plants) (Peroutka *et al.*, 2008).

No studies have assessed the possibility of endophytic fungi in *Nepenthes ampullaria* as potential PUR degraders. The aim of this study was to assess endophytic fungi isolated from *N. ampullaria* for their potential to degrade PUR, as well as to assess their enzymatic activity and changes to their proteome under PUR exposure.

MATERIALS AND METHODS

Endophytic fungi

The 150 fungal isolates used in this study were previously isolated from *Nepenthes ampullaria* by Wong *et al.*, (2015).

Screening for PUR-degrading Endophytes

Initial screening for capability to degrade PUR was carried out with modifications following the method of Crabbe *et al.*, (1994). In summary, fungal isolates were grown in the presence of an aqueous PUR dispersion (Bayer Material Science) to firstly screen for their ability to degrade PUR. A total of 150 fungal endophytes were grown on solid medium (PUR-1) containing 19 mM NaH_2PO_4, 33.5 mM K_2HPO_4, 7.6 mM $(NH_4)_2SO_4$, 2.5 mM, Na-Citrate, 250 µM $MgSO_4$, 19 µM thiamine, 0.05% casamino acids, 147 µM $FeCl_3 \cdot 6H_2O$, 14 µM $ZnCl_2 \cdot 4H_2O$, 12 µM $CoCl_2 \cdot 6H_2O$, 12 µM $Na_2MoO_4 \cdot 2H_2O$, 10 µM $CaCl_2 \cdot 2H_2O$, 11 µM $CuCl_2$, 12 µM $MnCl_2$, 12 µM H_2SO_4, and 1.8 mM HCl. A volume of 10 mL PUR and 80 g of agar was added to 1 L of this mixture. Polymer was added after the media was autoclaved. The PUR solid medium was poured into petri dishes, inoculated with 0.5 cm^3 plug of fungus grown on PDA using aseptic technique, sealed, and incubated at 25 °C. Triplicates were prepared for each endophyte and the negative control where no fungus was inoculated onto the medium. After 2-3 weeks of incubation, PUR clearance by endophytes was observed and the diameter of zone of clearance was measured and recorded.

Fungal identification and phylogenetic analysis

Positive fungal isolates were selected for microscopic and molecular identification. Fungal cultures were grown on PDA for 4 days at 25°C. The DNA extraction method followed the general method of Zhang *et al.*, (2010). A sterile toothpick was used to transfer a small amount of mycelia from the colony into 100 µL of pure water in a 1.5 mL microcentrifuge tube. The mixture was vortexed thoroughly and then centrifuged at 10, 000 × g for 1 min. After carefully discarding the supernatant using a pipette tip, 100 µL of TE buffer was added to the microcentrifuge tube. The mixture was finally incubated at 85°C in a water bath for 20 min. The crude extract contained genomic DNA and was stored at -20°C until use.

Each PCR prepared contain 2 µL of 10 × PCR buffer, 1.2 µL of dNTP mixture (2.5 mmol/L each), 0.8 µL of each primer (10l mol/L), 0.2 µL of Taq DNA polymerase (5 U µ/L) and 1 µl of genomic DNA in a total volume of 20 µL. The primer pairs used for amplifying full length ITS regions were ITS1 (5'-TCCGTAGGTGAACCTGCGGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3'). A PCR consisted of an initial denaturing step of 3 min at 95°C followed by 29 cycles (30 sec at 95°C, 30 sec at 47°C and 2 min at 75°C) finished by a final extension step at 72°C for 5 min. PCR products were resolved by electrophoresis through 1.0% agarose gels in TAE (2 mmol/L EDTA, 80

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mmol/L Tris-acetate, pH 8.0) and were visualized by staining with ethidium bromide. Sequencing was performed at Beijing Genomics Institute, BGI, Hong Kong. Endophyte sequences were aligned to organisms present in the Genbank database using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). The sequences obtained were analyzed against the NCBI (USA) database (Zhang *et al*., 2000) and a phylogenetic tree was constructed from genetic distance and bootstrap values calculated using MEGA 6 (Tamura *et al*., 2013). Sequences have been deposited with the NCBI GenBank database under accession numbers KR013146-KR013151.

Sole carbon source assay

The fungal isolates SBF4, SBF1, and SBF3 which displayed the top three highest activity during the initial screening were subjected to further tests. Isolates SBF4, SBF1, and SBF3 were grown on PUR without other carbon sources (PUR-2). PUR-2 liquid medium was prepared using the same ingredients as the solid PUR-1 media, but without sodium citrate, thiamine, casamino acids, and agar. PUR-2 liquid medium was added to sterile conical flasks of 250 mL and inoculated with three plugs of 0.5 cm^3 fungus grown on PDA. Triplicates were prepared for each test isolate. After a month of incubation at 25°C, culture flasks were observed for a visual change of the media. The absorbance of the triplicate cultures was measured on a Varian Cary 50 UV-Visible Spectrophotometer at wavelength of 600 nm which was blank with PUR-2 liquid medium without PUR.

Enzyme assay

The top two fungal strains SBF4 and SBF1 were inoculated in 100 mL of PUR-2 liquid medium with 1% PUR, and incubated at 25°C for 30 days. Cultures were centrifuged at 10 000 rpm for 10 min. The esterase assay was carried out in triplicates following the method of Kordel *et al*., (1991). In brief, *p*-nitrophenyl acetate was used as substrate for esterase. The 2.0 mM *p*-nitrophenyl acetate was dissolved in 10 mL 2-propanol and mixed with 50 mM potassium phosphate buffer of pH 7. A volume of 1 mL supernatant was added to 9 mL of the substrate emulsion and mixed. The optical density at 410 nm was monitored and recorded for 0 to 16 min against a blank without enzyme using a Varian Cary 50 UV-Visible Spectrophotometer. One unit of enzyme activity was defined as the amount of substrate forming 1 µmol of *p*-nitrophenol per min. The concentration of *p*-nitrophenol of isolate SBF4 and SBF1 was determined by a comparison to a standard curve of known *p*-nitrophenol concentrations.

Proteome study

Two growing conditions for SBF4 and SBF1 were used to study the changes in abundance of proteins; liquid culture media without 1% PUR (control) and with 1% PUR (treated). Triplicates were prepared and the inoculated flasks were incubated at 25°C for 3 weeks. The cell biomass was collected and used for protein extraction.

Fungal cells of SBF4 and SBF1 from both control and treated conditions were ground in liquid nitrogen using a cooled mortar and pestle. Protein extraction was carried out by protein precipitation using trichloroacetic acid (TCA) acetone following the general method of Natarajan *et al.* (2005).

The isoelectric focusing (IEF) was then carried out by loading the samples to 13 cm Immobiline™ DryStrip gels (IPG), with a non-linear pH 3-10 gradient (GE Healthcare) following the instruction manual of a PROTEAN® i12™ IEF Cell (Bio-Rad) with modifications. Electrophoresis was carried out using SE 600 Ruby Standard Dual Cooled Vertical System (GE Healthcare) at constant 20 mA and 20°C until the bromophenol blue dye front migrated 2 cm from the top. After 3-4 h of electrophoresis, the proteins on gels were fixed overnight in water containing 10% (v/v) acetic acid, and 50% (v/v) ethanol on a rocking platform at low speed. The gels were finally rinsed three times in Milli-Q water and resolved proteins on gels were stained using Pierce® Silver Stain Kit for Mass Spectrometry (Thermo Scientific) following the manufacturer's instructions. Cultures exposed to the same experimental conditions were performed in triplicates.

GS-800 Calibrated Densitometer (Bio-Rad) was used to scan the gel images after SDS-PAGE. The scanned gel images were analyzed using PDQuest 2-D analysis software (Bio-Rad). Both quantitative and statistical analyses with Progenesis SameSpots software (Nonlinear Dynamics) version 15 and t-test ANOVA ($p < 0.05$) was employed to determine the protein spots that showed significant change between the control and treated samples. Normalization of protein spot intensities were done to account for differences in emission levels between images using the 'total spot volume' method. This method involved each protein spot being expressed as a percentage of the total spot volume on that gel, and a background subtraction was performed following supplier's recommendations. The differences in protein spots among experimental treatments were identified by gel to gel matching. All matched spots were detected on at least two gels from each set of three gels. Data expressed in this manner is independent of possible errors that may occur due to minute difference in protein loading.

For identification, protein spots with fold changes of >2.0 were firstly excised using EXQuest Spot Cutter system controlled through Quantity One®1-D analysis software (Bio-Rad). Proteins were destained following procedures from Pierce® Silver Stain Kit for Mass Spectrometry (Thermo Scientific) and dried by speed vacuum centrifugation for 30 min. Dried peptides were treated with trypsin (12.5 mg/mL) in 25 mM NH_4HCO_3 in 10% ACN at 25°C overnight. All the peptides solution were dried and stored at -80°C until analysis by mass spectrometry. Peptides were analyzed by MALDI-TOF MS (AB SCIEX, Framingham, MA). By using the

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reflectron positive ion mode with an average of 4000 laser shots per spectrum, MALDI-TOF mass spectra were acquired. The TOF/TOF tandem MS fragmentation spectra for each sample were also acquired with an average of 4000 laser shots per fragmentation spectrum on each of the 7-10 most abundant ions present in each sample, eliminating trypsin autolytic peptides and other identified background ions.

A GPS Explorer workstation equipped with the MASCOT engine (Matrix science) were employed for both the resulting peptide mass spectra and associated fragmentation spectra to search the non-redundant database of the NCBI. Protein molecular weight or isoelectric point were not restricted during the search, with flexible carbamidomethylation of cysteine and oxidation of methione residues and with one allowed missed cleavage site. A protein score C.I.% or ion C.I.% of more than 95 was considered significant proteins. Identified proteins were tabulated with their functions.

RESULTS

Fungal identification and phylogenetic analysis

A total of 150 endophytic fungal isolates were assessed for their ability to degrade PUR and only 9 isolates (SBF4, SBF1, SBF3, SBF7, SBF8, SBF5, SBF6, SBF9, and SBF 2) displayed positive results - zones of clearance (Table 1). A microscopic examination of the active endophytic isolates revealed that they produced spores of four euseptate, pigmented median cells with three apical appendages, and a basal appendage (an example from SBF4 is shown in **Error! Reference source not found.**) which suggests that the isolates are related to *Pestalotiopsis* (Jeewon *et al.*, 2002). This was supported by Jeewon *et al.* (2002), where *Pestalotiopsis* has been characterized by spores that have slightly curved conidia bearing four to five-euseptate and pigmented median cells with two to four tubular extensions from the apical cell, and a centric basal appendage that arises from the basal cell. Pigmentation was also observed in the median cells of all the isolates and according to Griffiths and Swart (1974), this was due to the deposition of melanin molecules within the matrix of cell and the differences in pigmentation were of taxanomic value. The phylogenetic analysis supported this and revealed that all of the 9 active fungal endophytes belonged to the *Pestalotiopsis* genus (**Error! Reference source not found.)**.

Sole carbon source and enzyme assay

SBF4, SBF1, and SBF3 displayed the largest zones of clearance during the initial screening (32.7 – 36.0 mm; Table 1). All three isolates were able to utilize PUR as sole carbon source as indicated by the changes in absorbance from 0.362 (day 0) to 0.229 (day 30; see **Error! Reference source not found.**). This was also supported by an observed change in transparency of the liquid medium from chalky to almost transparent during the sole carbon source assay (**Error! Reference source not found.**). Clearance in both solid and liquid medium is suggestive of an enzymatic activity and cell-free supernatants of the top two PUR-degrading fungi (SBF4 and SBF1) were measured. Esterase activity was detected in both isolates' culture supernatant after a month of incubation. The concentrations of the end product of the enzymatic reaction (*p*-nitrophenol), and calculated enzyme activity for SBF4 and SBF1 were 10.27±0.6 μM, 1,850.4±100.1 U/mL and 9.47±0.1 μM, 1,710.2±161.7 U/mL; respectively. Isolate SBF4 displayed a slightly higher (albeit statistically not significant) enzymatic activity compared to SBF1.

Proteomic

Proteins of fungal isolates SBF4 and SBF1 were extracted to investigate the changes in the abundance of proteins when treated with PUR. Our analysis revealed that there were 431 protein spots detected on all triplicate gels of control and treated samples and when individual proteins present in the 2-DE gels were analyzed, 45 protein spots changed in abundance between the control and treated samples. The mean percentage volume of protein spots and *t* test was employed ($p < 0.05$), and among the 45 protein spots that were detected with altered abundance, 10 proteins spots showed fold changes of more than 2.0. These proteins were subsequently identified for their functions using mass spectrometry approach and among the 10 protein spots, 7 were metabolic enzymes, 1 involved in generation of energy while two others were without any known functions (Table).

Table 1: Average zone of PUR clearance of each fungal isolates measured in mm with standard deviation.

Isolate	Zone of clearance (mm)
SBF4 [GenBank accession number KR013146]	36.0 ± 1.7
SBF1 [GenBank accession number KR013143]	35.3 ± 0.6
SBF3 [GenBank accession number KR013145]	32.7 ± 0.6
SBF7 [GenBank accession number KR013149]	30.7 ± 1.2
SBF8 [GenBank accession number KR013150]	30.3 ± 0.6
SBF5 [GenBank accession number KR013147]	30.0 ± 0.6
SBF6 [GenBank accession number KR013148]	29.7 ± 0.6
SBF9 [GenBank accession number KR013151]	28.0 ± 0.6
SBF2 [GenBank accession number KR013144]	15.0 ± 0.6

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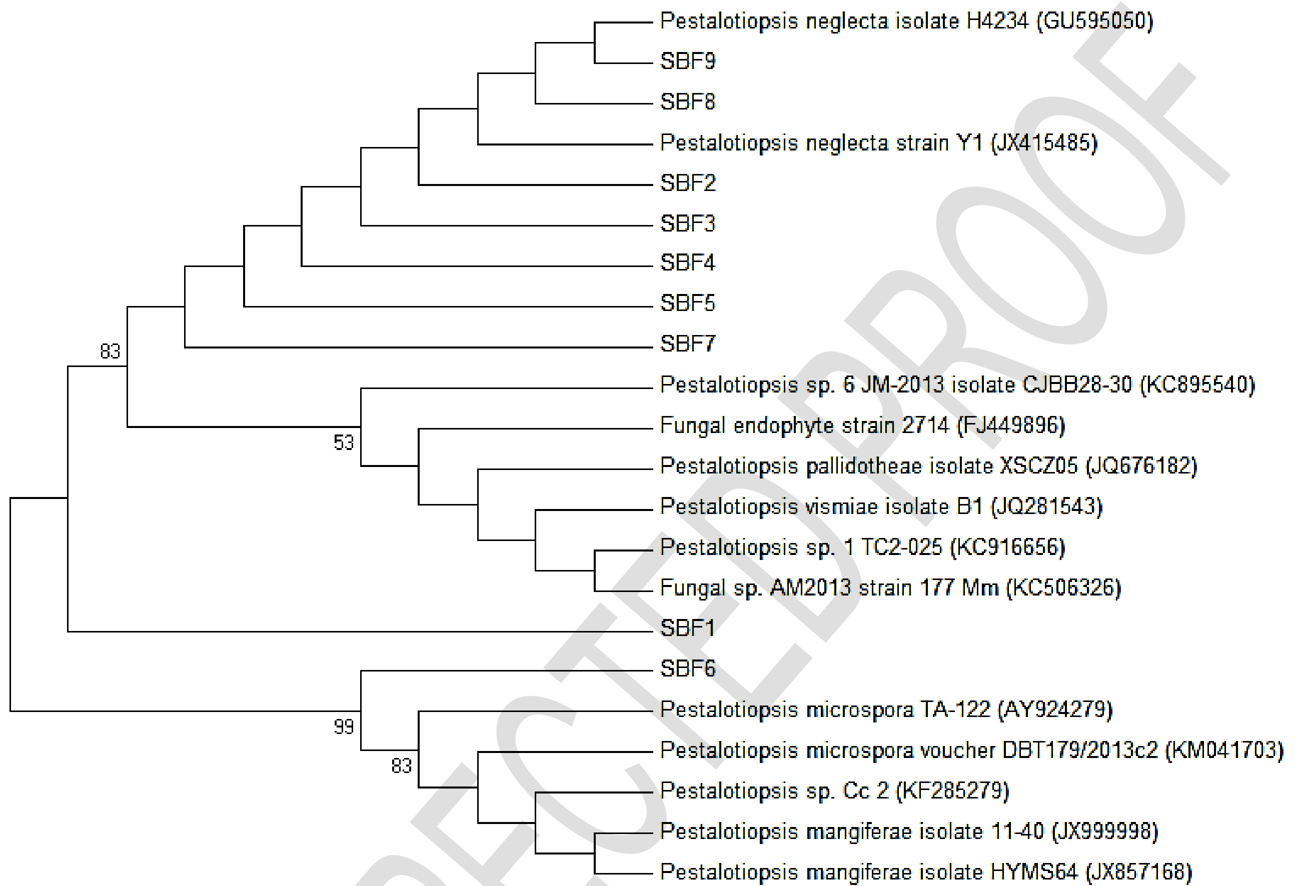
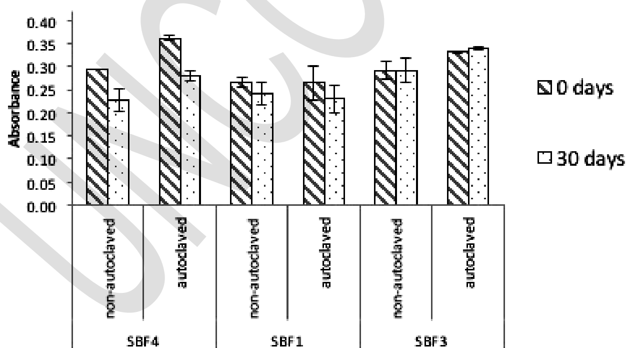
Error! Reference source not found. Microscopic view of most active PUR-degrading endophytic isolate (400× magnification).



Error! Reference source not found.: Internal transcribed spacer (ITS) gene-based phylogenetic tree representing fungal sequences found in fungal isolates, SBF. The phylogenetic tree was generated with the neighbour-joining method. Bootstrap values generated from 1000 replicates are shown above the branches. Accession numbers for the reference sequences are indicated.



Error! Reference source not found.: PUR degradation by active fungal endophytes. Cultures contain PUR-2 minimal media and inoculated with fungal inoculums. Error bars represent the standard deviation of average value of absorbance.

Error! Reference source not found.: Change of medium from chalky to almost transparent with fungal biomass.

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Table 2: List of identified proteins whose abundance were altered when isolate SBF4 and SBF1 were treated with PUR.

Spot ID	Accession	Protein	FC[a]	MW[b]	pI[c]	Protein Score	Organism	Biological Process[d]
195	FEN1_CANAL	Flap endonuclease 1	2.7	42,630	9.3	58	*Candica albicans*	DNA replication
218	ATPG_MOUSE	ATP synthase subunit gamma	2.4	32,979	9.6	48	*Mus musculus*	ATP binding
449	RL2_ACHLI	50S ribosomal protein L2	4.6	30,163	11.3	49	*Archoleplasma laidlawii*	Peptide bond formation
453	PPR61_ARATH	Uncharacterized protein	2.4	65,610	9.1	59		RNA activity
454	SYH_XYLF2	Histidine-tRNA ligase	2.2	52,321	5.6	74	*Xylella fastidiosa*	RNA editing
491	IFNT_OVIMO	Inteferon tau	3.0	22,697	6.5	45	*Ovibos moschatus*	Anti-viral, signaling activity
493	MT1_FESRU	Metallothionein-like protein	3.0	7,775	4.9	16	*Festuca rubra*	Heavy metal binding
652	Y3198_GEOLS	Uncharacterized protein	2.1	18,225	8.9	61	*Geobacter lovleyi*	Nucleotide binding
890	MURQ_ECO81	N-acetylmuramic acid 6-phosphate etherase	2.6	31,511	5.4	64	*Escherichia coli*	Cell wall recycling
1440	LEUC_THEP3	3-isopropylmalare dehydratase large subunit	2.1	45,737	5.8	55	*Thermoanaerobacter pseudethanolicus*	Leucine biosynthesis

[a]fold change; [b]molecular weight; [c]isoelectric point; [d]http://www.uniprot.org

DISCUSSION

All nine PUR-degrading fungal strains isolated from *N. ampullaria* samples represent only 1 genus, *Pestalotiopsis*. The *Pestalotiopsis* genus is known to be a good source for numerous valuable secondary metabolites. Endophytic *Pestalotiopsis* species isolated from medicinal plants exhibited anitioxidant, antihypersensitive, and antibacterial activities that demonstrated the potential of *Pestalotiopsis* extracts as therapeutic targets (Tejesvi *et al*., 2008). Besides the production of bioactive compounds, *Pestalotiopsis* have been shown to be able to biosorb various heavy metals (Choo *et al*., 2015).

There are however, very few studies available of endophytic fungi capable of degrading PUR plastics. Russell *et al.* (2011) found that *Pestalotiopsis microspora* was able to degrade the synthetic polyester polyurethane efficiently in both solid and liquid suspensions, and suggested that serine hydrolase is the enzyme produced by *P. microspora* that is responsible for PUR degradation. There are no previous reports yet of the genus *Pestalotiopsis* from *N. ampullaria* that could degrade PUR nor on their proteins.

In our study, the *Pestalotiopsis* related top three SBF4, SBF1, and SBF3 isolates also showed to have positive results on both sole carbon source and enzyme assay. The cell-free supernatant was autoclaved and non-autoclaved to test for inhibition of enzymatic activity responsible for the degradation activity of PUR. However, there were only small detectable differences between the autoclaved and non-autoclaved on degradation activity. Enzymatic activity was still detected in this study and this suggests the presence of enzyme responsible for PUR degradation is not affected by high heat treatment. Russell *et al.* (2011) also revealed that heated filtrates at 60°C and 80°C did not affect the degradation of substrate PUR. Studies conducted by Maeda *et al.* (2005) discovered that *Aspergillus* could digest plastics with specific activities up to 11,000 U/mL. The enzymatic activity obtained in this study is not as high, however, this study did not carry out any optimization of the process and further studies can be done with a focus on testing more parameters including the different temperatures and pH to achieve the best optimum enzymatic activity.

Proteins of both fungal isolate SBF4 and SBF1 in this study were altered in abundance when treated with PUR. The significantly altered proteins were identified to be involved in either metabolic functions or ATP generation. While there was no obvious protein involved in plastic degradation, the increased expression (3 times) of a metallothionein-like protein is remarkable as these proteins are usually involved in detoxification of heavy metals and maintaining the homeostasis of essential metal ions (Suhy *et al*., 1999). There may be a role for metallothionein-like proteins in the degradation of PUR as well. However, further studies are needed to identify the exact enzymes and proteins involved in the degradation of PUR.

CONCLUSION

In conclusion, endophytic fungi related to *Pestalotiopsis* isolated from *N. ampullaria* show promising PUR-degrading activity. *N. ampullaria* is a good novel source for *Pestalotiopsis* which in turn could in future be developed as bioremediation agents for E-waste, particularly considering that the same genus has also shown heavy metal absorbing capacities (Choo *et al*., 2015).

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