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Isolation and Characterization of Coprophilous Cellulolytic Fungi from Asian Elephant (*Elephas maximus*) Dung

Ahmadu Ali Farouq[1], *Dzulkefly Kuang Abdullah[1,4], Foo Hooi-Ling[2], Norhafizah Abdullah[3]

1. Laboratory of Industrial Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang. Selangor. Malaysia.
2. Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang. Selangor. Malaysia.
3. Department of Chemical and Environmental Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang. Selangor. Malaysia.
4. Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang. Selangor. Malaysia.

* E-mail of the corresponding author: dzul2240@gmail.com

Abstract
A lot of work has been done on isolation of cellulolytic fungi from the natural environment, but no such work was done on Asian elephant dung in Malaysia. Fungi that grow on elephant dung which is full of fibres may serve as a source of potential cellulase enzymes. Commercial cellulase enzymes used for the hydrolysis of lignocellulose biomass are not only expensive, but may as well hinder progress in the bioethanol industry. In this study, eight new fungi were isolated from Asian elephant dung sourced from Malaysian forest reserve. The fungi were identified morphologically and by molecular assay. The sequences of the fungi were deposited in the Gen Bank NCBI and were assigned accession numbers. Phylogenetic tree of the fungi was constructed to show similarities of the new fungi to known strains. The fungi were tested for cellulolytic potential using carboxymethyl cellulose (CMC). *Trichoderma aureoviride* strain UPM 09 (JN811061) and *Fusarium equiseti* strain UPM 09 (JN811063) proved to be potential cellulolytic fungi. This study shows that nature harbors the best cellulolytic fungi for biotechnological applications yet to be exploited.
Keywords: cellulolytic, fungi, cellulase, lignocelluloses, biomass, Trichoderma aureoviride, Fusarium equiseti

1. Introduction

Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrates in particular (Lynd *et al*., 2002). Coprophilous fungi are the dung-loving fungi (copro: dung; philous: loving). A lot of work on isolation of cellulolytic fungi from the environment has been done (Jahangeer *et al*., 2005). Sohail *et al.* (2009) isolated a cellulolytic *Aspergillus niger* species from natural environment (soil, air and infected plant). However, no such work was done on elephant dung which is mainly composed of cellulose fibers. Cellulose is a linear polymer of glucose units linked together by ß-1, 4-glucosidic bonds and forms the major component of plant biomass; a variety of fungi and bacteria can convert this insoluble substrate to soluble compounds by elaborating cellulases enzyme, commercial products of which make bioethanol production extremely costly (Sohail *et al*., 2009). This study is undertaken in order to source for cheaper cellulases from nature. Fungi that grow on elephant dung are believed to be potential cellulase enzyme producers that may make bioethanol production less expensive. The aim of this study, therefore, is to isolate fungi from Asian elephant (*Elephas maximus*) dung, identify, characterize them phylogenetically and test their cellulolytic potential using cellulose substrate.

2. Materials and Methods

2.1 Collection of Asian Elephant (E. maximus) Dung Samples

Samples of Asian elephant (*E. maximus*) dung were collected from the Royal Belum Park in the Perak State of Malaysia. Fresh samples of the dung were collected in sterile containers and transported to the laboratory for culture preparation.

2.2 Sample Collection Site

The Royal Belum State Park was established in the year 2000. It is situated in the northern state of Perak in Malaysia. Its geographical coordinates are 5° 25′ 0″ North, 101° 8′ 0″ East. The park is a habitat for wild life including Asian elephants.

2.3 Preparation of Elephant Dung

One gram (1g) of the elephant dung was weighed and put in a sterile test tube containing 10 milliliters of sterile

distilled water to make a suspension or slurry. Serial dilution was carried out by transferring 1ml of the suspension into the first test tube and to the next, and on up to the 9th of the nine sterile test tubes arranged in a rack each containing 9 ml of sterile distilled water (Tripathi, 2006) before inoculation on potato dextrose agar medium.

2.4 ***Preparation of Medium and Inoculation***

Potato dextrose agar medium was prepared as described by the manufacturer (Oxoid) by dissolving 39 g in 1 liter of distilled water. The medium was sterilized in an autoclave at 121ºC for 15 mins. Tetracycline (125 mg) was incorporated in the medium to inhibit growth of bacteria. Using streak plate method, the diluted dung suspension was inoculated using an inoculating loop on Potato Dextrose Agar medium and incubated at 30ºC for 4 to 7days. The colonies of the fungi were then sub cultured on potato dextrose agar medium to obtain pure colonies. The fungal cultures were routinely sub cultured on fresh potato dextrose agar medium using streak and pour plate methods in order to obtain pure colonies.

2.5 ***Morphological Identification and Characterization of Fungi***

The fungal colonies observed were identified using light microscopy (×40, ×100) by staining with phenolic rose Bengal or cotton blue reagent (Tripathi, 2006).The various characteristics of the hyphae, mycelia and conidia were studied to aid in identification of the fungi. DNA extraction, PCR Amplification and sequencing were used to identify the fungi as described by Pant and Adholeya (2007).

2.6 ***Molecular Identification of Native Fungi***

Following the instructions of the manufacturer (Epicenter®), the Genomic DNA of the fungi was extracted. About 1-5 mg each of fresh fungal mycelia was diluted with a solution containing 1 µl of 50 µg/µl Proteinase K and 300 µl of Tissue and Cell Lysis Solution and mixed thoroughly before homogenizing. The tubes were incubated at 65ºC for 15 minutes and vortexed every 5 minutes. The samples were placed on ice for 3-5 minutes. 175 µl of MPC protein precipitation reagent was added to 300 µl of lysed sample and vortexed vigorously for 10 seconds. The debris was pelleted by centrifugation at 4ºC for 10 minutes at 10,000 x g in a micro centrifuge. The supernatant was transferred to a clean micro centrifuge tube and discarded the pellet. About 500 µl of isopropanol was added to the recovered supernatant. The tubes were inverted 30-40 times. The total nucleic acids were pelleted by centrifugation at 4ºC for 10 minutes in a micro centrifuge. The isopropanol was carefully poured off without dislodging the total nucleic acid pellet and rinsed twice with 70% ethanol, being careful to not dislodge the total nucleic acid pellet. The residual ethanol was removed with a pipette. The total nucleic acids were resuspended in 35 µl of TE Buffer. Following extraction, genomic DNA concentration was measured using Biophotometer (Eppendorf, Germany).This was achieved by taking the sample's optical density (OD) at a wavelength of 260 nm. One OD was equivalent to 50 µg / mL of DNA. On the other hand, the sample's purity was determined by taking the ratio of 260 nm and 280 nm optical densities. Samples with ratio between 1.8 nm and 2.0 nm were considered pure.

2.7 ***PCR Amplification and Sequencing***

Two universal oligonucleotide primers were used for polymerase chain amplification. They were the ITS-1 forward primer (5' TCC GTA GGT GAA CCT GCG G3') and the ITS-4 reverse primer (5' GCT GCG TTC TTC TTG ATC GAT GC 3'). The primers were developed based on descriptions by White *et al*. (1990) and were supplied by 1st Base Laboratories, Malaysia. Amplification reactions were conducted in 50µl reaction mixture containing a final concentration l µl of 10 X PCR buffer (NovaTaq®), 4µl of $MgCl_2$, (25mM) 0.5µl of Nova *Taq* polymerase (100U), 1µl of dNTP (10Mm) and 4µl of genomic DNA. All the reagents above were obtained from Novagen Lab, USA. Polymerase Chain Reaction (PCR) amplifications were performed using the BIOMETRA Tpersonal/Tprofessional Thermo cycler (Germany) with an initial denaturation of 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 90 secs and extension at 72°C for 90 secs, with a final extension step at 72°C for 7 minutes. After amplification, the products were used immediately or stored at 4ºC until required (Saitoh *et al.,* 2006). Aliquots of 5 µl of each PCR product were subjected to electrophoresis on a 1.8% horizontal agarose gel (Promega, USA) in a 1 X TBE buffer at 70V for 90 mins, depending on the size of the amplified fragment from the primer. The gel was stained in 1µl of Gel Red for 15 to 20 minutes. A 1 kb marker (Fermentas) was used as molecular weight standard to estimate the size of the ITS 1 regions amplified. The gel was photographed by using an Ultraviolet transilluminator and the ITS products were photographed by using the UVDI Analyser (Major Science, TAIWAN).

2.8 ***Molecular Sequence Analysis***

The PCR products were sequenced by 1st Base Laboratories (Malaysia). The resultant nucleotide sequences were assembled and manually edited with Bio Edit software, version 7.0.9. Furthermore, the nucleotide sequences were subjected to sequence homology search using BLAST tool (available at http:/blast.ncbi.nlm.nih.gov). In performing the search, sequence with lower *E*- value score of $<10^{-3}$ and $\geq$ 70% were considered homologous. Nucleotide sequences generated in this study were also deposited in NCBI- Gen Bank database, USA and were assigned accession numbers. The phylogenetic tree was constructed using MEGA 5 Software (Tamura *et al*.,

2011).

2.9 ***Screening of Native Fungi for cellulolytic Activity***

Cellulolytic Basal Medium (CBM) medium as described by Pointing (1999) was prepared. Carboxymethylcellulose CMC (2 % w/v) and 1.6 % w/v agar were incorporated into the medium and autoclaved. This was aseptically transferred to Petri dishes (agar was cooled until viscous and gently mixed before pouring to ensure uniform distribution of cellulose in the agar medium). Then the agar was inoculated with the test fungus. Incubation was done at 25ºC in darkness and was examined daily for 10 days. The plates were flooded with 2 % w/v aqueous Congo red (C.I. 22120) and left for15 minutes. The stain was poured off and washed the agar surface with distilled water. The plates flooded were with IM NaCI to destain for 15 minutes. Cellulolysis was assessed based on clearance zones of the opaque agar around growing colonies.

3. Results and Discussion

3.1 Characterization of Isolated fungi

Eight new fungi were isolated in this study. The fungi were deposited in the culture collection of Universiti Putra Malaysia (UPMC) while their sequences were deposited in the Gen Bank NCBI database (USA), and were assigned accession numbers (prefixed UPMC and JN, respectively). These include *Trichoderma aureoviride* UPM 09 (JN811061) (UPMC 389), *Wickerhammomyces anomalus* UPM 02-09 (JN811059) (UPMC 394), *Wickerhammomyces anomalus* (*Pichia anomala*) UPM 01-09 (JN811060) (UPMC 394), *Aureobasidium pullulans* UPM 09 (JN811062) (UPMC397), *Gibberella intermedia* UPM-09 (JN811064) (UPMC390), *Fusarium equiseti* UPM 09 (JN811063) (UPMC391), *Rhizomucor variabilis* UPM 09 (JN806137) (UPMC 392) and *Schizophyllum commune* UPM 09 (JN811058) (UPMC395). The colony and microscopic morphology of some of the fungal isolates are presented in Table 1. All the fungi are natural fungi since the dung samples were collected from a site situated deep in the rainforest jungle in sterile containers. The PCR amplified fragments of the DNA of the isolates are presented in Figs. 1 and 2. Their sizes range between 200 to 320 bp.

3.2 Phylogenetic tree of the Native Fungi

The phylogenetic tree of the native fungi isolated in this study is presented in Fig. 3. The tree shows the relationship between the fungi isolated in this study (in bold) and other related species isolated elsewhere with 100 % resemblance. With respect to DNA identity, the sequence similarity among the local isolates ranges from 33% to 100%. *W. anomalus* (*P. anomala*) UPM 01-09 and *W. anomalus* UPM 02-09 are 100% similar (Table 2). *F. equiseti* UPM 09 and *G. intermedia* UPM 09 are grouped under the same cluster. Likewise, *W. anomalus* (*P. anomala*) UPM 01-09 and *W. anomalus* UPM 02-09 are also grouped under the same cluster. While each cluster has one of the local isolates distributed evenly among the six clusters. This is confirmed by sequence identity matrix (Table 2). The phylogenetic tree confirms that all the species are fungi and are also evolutionary related to other fungal species. This implies that the fungi are closely related can also be potentially cellulolytic.

3.3 Screening for cellulolytic activity

The result of the cellulolytic screening is presented in Table 3 where *Trichoderma aureoviride* UPM 09 had the highest cellulolytic activity (4.0 mm) and *A. pullulans* strain UPM 09 had the lowest cellulolytic activity (0.75 mm). This result shows that *T. aureoviride* UPM 09 had the highest potential for cellulolytic activity.

4. Conclusion

The Asian Elephant (*Elephas maximus*) dung was found to be a good source of new cellulolytic fungi which can be used as a cheap source of cellulase enzyme in the bioethanol industry which may ultimately be a substitute for the expensive commercial cellulase enzyme. *Trichoderma aureoviride* strain UPM 09 (JN811061) and *Fusarium equiseti* strain UPM 09 (JN811063) proved to be potential cellulolytic fungi. This study shows that nature harbors the best cellulolytic fungi for biotechnological applications yet to be exploited.

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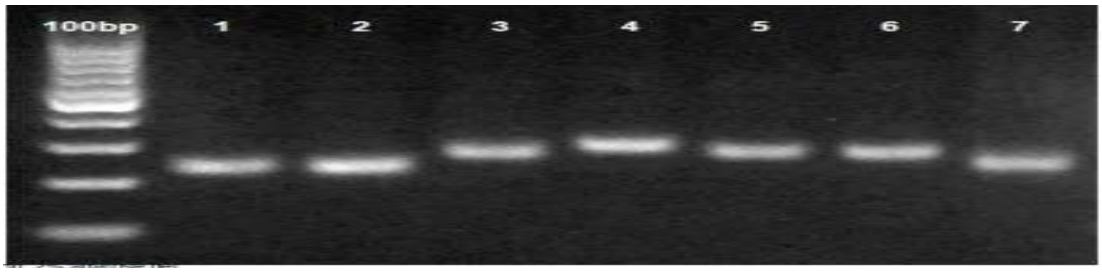

Figure1. PCR amplified fragments of Genomic DNA of the Native fungi strains-L=100bp Ladder/Marker, Lane 1= *F. equiseti* strain UPM 09 (JN811063), Lane 2= *Gibberella intermedia* strain UPM 09(JN81164), Lane 3= *W. anomalus* strain UPM 09- 02 (JN811059), Lane 4 = *T. aureoviride* strain UPM 09 (JN811061), Lane 5= *Rhizomucor variabilis* strain (JN806137), Lane 6= *Aureobasidium pullulans* strain UPM 09(JN811062) and Lane 7. *Schizophyllum commune* strain UPM 09 (JN811058).

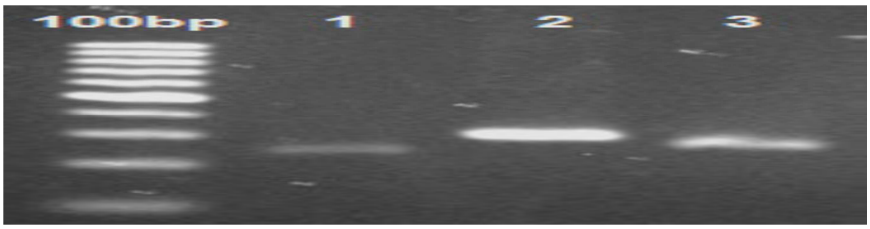

Figure 2. PCR amplified fragments of Genomic DNA of the Native fungi strains-L=100bp Ladder/Marker, Lane 1= *Gibberella intermedia* strain UPM 09 JN811064, Lane 2= *Rhizomucor variabilis* strain (JN806137) and Lane 3= *W. anomalus* (*P. anomala*) UPM 09- 01 (JN811059).

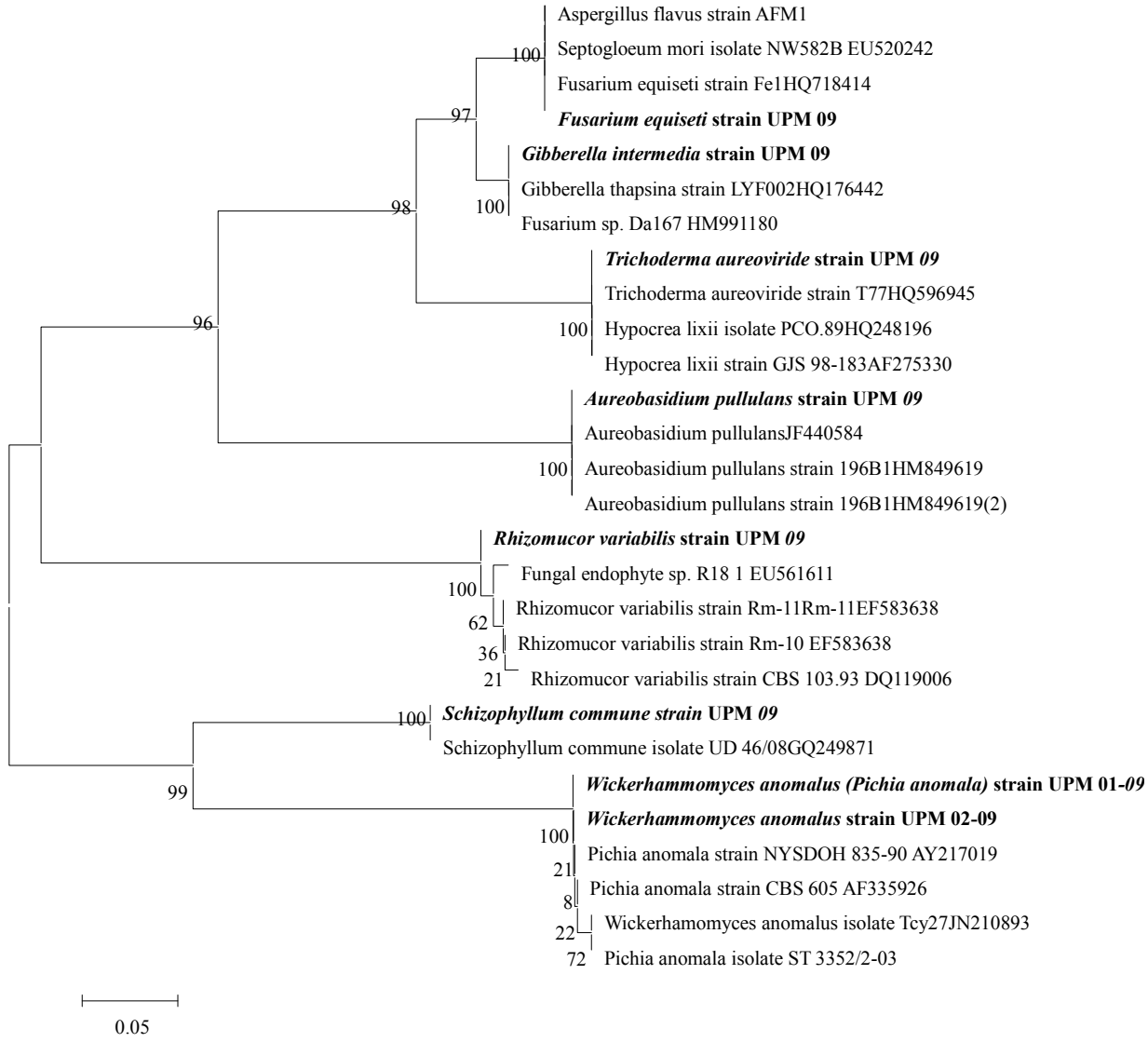

Figure 3. Phylogenic tree of the native fungi isolated from Asian Elephant (*E. maximus*) dung. Phylogenetic tree was obtained using the neighbour-joining algorithm, reflecting the relationships of 16S rRNA gene sequences. Branches also supported by parsimony and maximum-likelihood. Percentages above branches correspond to 1000 bootstrap replicates using the neighbour-joining algorithm. Malaysian isolates are indicated in bold.

Table 1. Morphology of the Fungal Isolates from Asian Elephant dung

Fungi	Colony Morphology	Microscopic Morphology
a). *F. equiseti* strain UPM 09 (Ascomycota)	whitish, pinkish background with light periphery, remain white	septate hyphae, canoe-shaped, multiseptate unbranched
b). *T. aureoviride* strain UPM 09 (Ascomycota)	white fluff at first, compact wooly, later green	conidiophores long, tufted, compact
c). *W. anomala (P. anomala)* strain UPM 09 (Ascomycota)	milky, cream-colored	spherical, elliptical, oblong cells
d). *W. anomalus* strain UPM 09 (Ascomycota)	white tufts, compact	elliptical, oblong cells, smooth elliptical, oblong cells, hyphae, septate; conidia ellipsoidal one-celled
e). *A. Pullulans* UPM 09 (Ascomycota)	smooth faint-pink slimy spores	
f). *S. commune* UPM 09 (Basidiomycota)	upper surface covered with small hairs, white to grayish smooth wooly tufts	cylindrical to elliptical, smooth spores
g). *R. variabilis* strain UPM 09 (Zygomycota)	Wooly, whitish thread-like, mucoid mycelia	bearing sporangiospores on sporangia
h).*G. intermedia* UPM-09 (JN811064) (UPMC390)	whitish, light pinkish with light periphery, remain white	septate hyphae, canoe-shaped, multiseptate unbranched

Table 2. Sequence Identity Matrix of Fungal isolates

Sequences	*R. variabilis* UPM 09	*W. anomalus* UPM01-09	*F. equiseti* UPM 09	*S. commune* UPM 09	*W. anomalus* UPM 02-09	*T. aureoviride* UPM 09	*A. pullulans* UPM09	*G. intermedia* UPM 01-09
R. variabilis								
UPM 09	ID	0.491	0.491	0.327	0.491	0.498	0.479	0.498
W. anomalus								
UPM01-09	0.491	ID	0.536	0.421	1	0.516	0.547	0.531
F. equiseti								
UPM09	0.491	0.536	ID	0.354	0.536	0.691	0.62	0.94
S. commune								
UPM09	0.327	0.421	0.354	ID	0.421	0.347	0.339	0.348
W. anomalus								
UPM02-09	0.491	1	0.536	0.421	ID	0.516	0.547	0.531
T. aureoviride								
UPM 09	0.498	0.516	0.691	0.347	0.516	ID	0.565	0.705
A. pullulans								
UPM09	0.479	0.547	0.62	0.339	0.547	0.565	ID	0.645
G. intermedia								
UPM01-09	0.498	0.531	0.94	0.348	0.531	0.705	0.645	ID

Table 3. The ratio between the Clearing zone (mm) and colony diameter (mm) grown on cellulose agar medium

Fungus	Clearing zone (mm)	Colony diameter (mm)	Ratio of Clearing zone/Diameter (mm)	Clearing zone (mm)
T. aureoviride UPM 09 (JN811063)	4.0	1.0	4.0/1.0	4.0
F. equiseti strain UPM 09 (JN811061)	2.5	1.0	2.5/1	1.0
W. anomalus strainUPM02- 09 JN811059	ND	ND	ND	ND
A. Pullulans strain UPM 09 JN811062	3.0	4.0	3.0/4.0	0.75
S. commune strain UPM 09 JN811058	2.9	3.0	2.9/3.0	0.97
R. variabilis strain UPM 09 JN806137				
W. anomalus (*P. anomala*) strain UPM01- 09 JN811059	ND	ND	ND	ND
	ND	ND	ND	ND

ND= Not Detectable; figures in mm represent average diameter of three zones for each fungus.