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Original Article

Antibacterial and lignocellulose-degrading enzyme activities of coprophilous fungi obtained from cow dung in Thailand

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Abstract

Twenty-seven coprophilous fungi, isolated from field-fed cow dung in an organic farm in Thailand, were identified using morphology and ITS barcode. A total of five genera viz. *Aspergillus*, *Hamigera*, *Paecilomyces*, *Penicillium*, and *Talaromyces* were identified with varying numbers and growth rates. These fungi were evaluated for their antibacterial properties against Gram-positive (*Bacillus subtilis*, *Kocuria rhizophila*, *Staphylococcus aureus* and *St. epidermidis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. *Pe. javanicum* NTD-SP2-01 and *Talaromyces* sp. NTD-SP5-48 exhibited activity against all bacteria when tested with agar plug diffusion method. *Talaromyces* sp. NTD-SP5-48 was particularly effective against Gram-negative bacteria. *As. terreus* NTD-NG1-05 displayed the highest activity against five bacterial strains, except *Ps. aeruginosa*. Notably, *As. terreus* NTD-NG1-05 and *Talaromyces* sp. NTD-SP5-48 demonstrated extended antibacterial activity in the agar disk diffusion method, with fermented broth (FB) showing superior inhibitory effects compared to mycelial extract (MY). Both isolates demonstrated significant antibacterial activity against *B. Subtilis*. Furthermore, all isolates exhibited significant antibacterial activity against *B. subtilis*, with a diffusion of 0.125 mg/disk. Only *Talaromyces* sp. NTD-SP5-48 (FB) displayed the highest inhibition activity against *Ps. aeruginosa*, with a diffusion of 1 mg/disk (100 mg/mL). In terms of enzyme activity, all isolates exhibited cellulase activity, with *Talaromyces* sp. showing the highest cellulase activity, followed by *As. terreus*. Laccase activity was only observed in the unidentified isolate NTD-SP5-34, while none of the isolates showed pectinase activity.

Keywords: Antimicrobial activity, Coprophilous fungi, Heat-resistant fungi, Lignocellulolytic enzymes

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Introduction

Fungi exhibiting a wide range of forms are found around the globe, and serve crucial functions as decomposers, mutualists, and parasites across various biomes. The estimated count of fungal species ranges from 2–11 million (Phukhamsakda et al., 2022). Hyde (2022) approximated that there are between 2.2 to 3.8 million fungal species based on host association, while high-throughput sequencing indicated a wider range of 11.7 to 13.2 million species. As of the latest update on February 27, 2024, the Index Fungorum database has reached a number of 618, 269 entries (https://www.speciesfungorum.org/Names/Names.asp). Fungi also are classified according to their ability to thrive in different temperature conditions: psychrophilic (below 20 °C), mesophilic (20–40 °C), thermophilic (at or above 20 °C with growth potential up to 60 to 62 °C), and thermotolerant (below 20 °C to ~55 °C) according to studies by Maheshwari et al., 2000; Brito De Oliveira et al., 2015; Witfeld et al., 2021. Certain fungi such as *Aspergillus fumigatus* and *As. terreus* exhibit thermophilic/thermotolerant characteristics and are also known for their heat resistance (Sharma et al., 2014; Witfeld et al., 2021). However, it is important to note that heat resistance is not found in all fungi, but rather in specific genera such as *Aspergillus*, *Byssochlamys*, *Eupenicillium*, *Fusarium*, *Mucor*, *Neosartorya*, *Paecilomyces*, *Penicillium*, *Rhizopus*, and *Talaromyces* (Piecková et al., 2019). Coprophilous fungi, also known as dung-loving fungi, thrive on herbivore dung as saprobes. These coprophilous fungi are primarily classified under the phyla Ascomycota, Basidiomycota, and the former Zygomycota, especially the Mucoromycota (Gryganskyi et al., 2023) though majority of these species are part of the Ascomycetes group (Guevara-Suarez et al., 2020; Mumpuni et al., 2021; Senanayake et al., 2022). Piasai and Manoch (2009) conducted a study on the diversity and distribution of coprophilous fungi in the dung of various wildlife and domestic animals such as barking deer, buffalo, cow, and elephant in Thailand. They discovered 49 isolates of Ascomycetes belonging to 16 different genera, including *Ascobolus*, *Cercophora*, *Chaetomium*, *Coprotus*, *Emericella*, *Eupenicillium*, *Eurotium*, *Gelasinospora*, *Hamigera*, *Podospora*, *Neosartorya*, *Saccobolus*, *Sordaria*, *Sporormiella*, *Talaromyces* and *Xylaria*. Mungai et al. (2011) similarly conducted a study on the taxonomy and diversity of coprophilous ascomycetes found on dung from Asiatic elephant, cattle, chicken, goat, and water buffalo in Thailand. The latter study identified a total of 48 isolates belonging to 11 fungal species, including common coprophilous species such as *A. immersus*, *Cercophora kalimpongensis*, *Sa. citrinus*, and *Sporormiella minima*. They are widely distributed in herbivorous animal dung in nature, where they decompose not only readily available sugars but also complex lignocellulosic components of plant cell walls, such as cellulose, hemicellulose, lignin, and pectin (Peterson et al., 2011; Makhuvele et al., 2017; Dicko et al., 2020; Mumpuni et al., 2021). Industries employ lignocellulose or agricultural residues as a primary source to manufacture a wide range of products such as chemicals, biomaterial, biofuels or biogas, pulp, and paper. Fungi-produced enzymes are used in industries related to food and beverage, pulp and paper, textiles, biomedicals (anticancer, antimicrobial, and antioxidant) as well as environmental protection (biodegradation, bioremediation, and decolorization) (El-Gendi et al., 2022). Numerous biotechnological procedures require high temperatures to facilitate reactions, thus making heat-resistant, thermophilic, and thermotolerant fungi essential components utilized in biochemical processes due to their proficiency in lignocellulose degradation and thermoresistant- or thermostable-enzyme production (di Piazza et al., 2020). Elsababty et al. (2015) identified several heat-resistant fungi, including *Arthrinium* sp., *As. nidulans*, *As. spinosus*, *H. avellanea*, *T. trachyspermus*, *T. barcinensis*, *T. ucrainicus*, and *Tr. asperellum*, capable of producing cellulolytic and pectinolytic enzymes. In a study conducted by Jasim et al. (2021), coprophilous fungi such as *Aspergillus* sp. (*As. niger*, *As. fumigatus*, *As. flavus*, *As. terrus*), *Chaetomium* sp., *Sordaria* sp., and *Podospora* sp. exhibited significant antimicrobial activity when tested against five pathogenic bacteria (*Enterobacter* sp., *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, and *Streptococcus* sp.) using disk diffusion assay. Bills et al. (2013) compiled data on coprophilous Ascomycota, which exhibit promising biological properties such as antibacterial, antifungal, and antitumor activities. Furthermore, Mohankumar and Savitha (2019) demonstrated that *Coprinopsis cinerea* (Basidiomycota) found in horse dung exhibited significant antibacterial activity against *St. aureus* and *Klebsiella pneumoniae*. However, the modern fungal taxonomy is primarily based on their

morphological features and the analysis of DNA sequences from the most conserved genes (Hawksworth, 2011). In a recent study by Mumpuni et al. (2021), nucleotide sequence data in ITS region was used to identify coprophilous fungi found in Indonesia at both the species and fungal generic level. The identified fungi included *Ceriporia lacerata*, *Lentinus squarrosulus*, *Trichosporon insectorum*, *Aspergillus* sp., *Fusarium* sp. and *Trichosporon* sp. Similarly, Jasim et al. (2021) used ITS region to verify the presence of coprophilous fungi (*Aspergillus* sp., *Chaetomium* sp., *Sordaria* sp., and *Podospora* sp.) in the dung of cows, buffalos, sheep, and camels in Basra. Desjardin and Perry (2017) reported *Panaeolus antillarum* (Basidiomycota) from wild elephant dung in Thailand, based on morphological and nucleotide sequence analyses of the ITS region. In this work, the focus was on the isolation of coprophilous fungi from cow dung aiming to explore the antibacterial and lignocellulosic enzyme activities of the secondary metabolites present.

Material and Methods

Collection, isolation, and culturing of heat-resistant coprophilous fungi

Freshly voided cow dung samples were collected from Rukkaset Farm, My Land New Theory Agriculture Learning Center (both organic farms in Bangkok and Suphan Buri, respectively) and from field-fed cows (a country farm in Suphan Buri) in Thailand. The samples were placed in plastic bags and promptly transported to the laboratory in an icebox. The fungi were isolated using a modified heat treatment method by Jesenská et al. (1992), as follows: 50 g cow dung was mixed with 50 mL of sterile distilled water in a 250 mL Erlenmeyer flask. The mixture was then heated in water bath at 60 °C for 30 minutes, with gentle mixing every 10 minutes. Subsequently, the sample was serially diluted with sterile distilled water until 10^{-3} dilution was achieved; then, 1 mL of each aliquot was plated onto sterile Petri dishes containing cow dung potato dextrose agar (CPDA) (100 g/L cow dung infuse, 100 g/L potato infusion, 10 g/L dextrose, 15 g/L agar supplemented with 1.25 g/L amoxicillin). These plates were then incubated at laboratory temperature (approximately 30±2 °C) for 3-5 days. Fungal colonies that appeared on the plates were aseptically transferred to fresh plates to obtain pure isolates. These pure culture isolates were then conserved in CPDA and potato dextrose agar (PDA) for further use.

Identification of coprophilous fungi

The fungal isolates were identified using microscope-based morphology and molecular analyses. Genomic DNA was extracted from fresh mycelium using a rapid DNA extraction method (Tangthirasunun and Poeaim, 2022). Molecular identification of the fungi was done based on nuclear ribosomal internal transcribed spacer (ITS), which was PCR amplified using either ITS1/ITS4 or ITS5/ITS4 primers (Toju et al., 2012). The PCR amplification process was conducted in a 25 µl final volume mixture, consisting of 14.3 µl nuclease-free water, 2.5 µl 10× standard Taq reaction buffer, 4 µl dNTP (1.25 mM each), 1 µl of each primer (5 µM), 0.2 µl Taq DNA polymerase (1 U/µl, BioLabs), and 2 µl DNA template. The PCR conditions were set as per the methodology outlined in the previous study (Tangthirasunun and Poeaim, 2022). Subsequently, the PCR products were detected through electrophoresis on a 1% agarose gel with 100 bp DNA ladder and then sequenced at Celemics, Inc. Korea using the Barcode taq sequencing (BTSeq) technique based on Next-Generation sequencing (NGS), Illumina Hiseq. The sequences were verified using BioEdit (version 7.2) and compared against the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLAST) program (www.ncbi.nlm.nih.gov/blast). The newly obtained sequences were deposited in GenBank under the GenBank accession number (Table 1).

Primary antibacterial activity screening by agar plug diffusion method

All fungal isolates were tested for antibacterial activity by the agar plug diffusion method of Balouiri et al. (2016). These isolates were tested against six human pathogenic bacteria, consisting of four Gram-positive (*Bacillus subtilis* TISTR 1248, *Kocuria rhizophila* (*Micrococcus luteus*) TISTR 2374, *St. aureus* TISTR 746 and *St. epidermidis* TISTR 2141) and two Gram-negative bacteria (*E. coli* TISTR 074 and *Pseudomonas aeruginosa* TISTR 2370). The bacterial strains were streaked on a nutrient agar (NA) plate and incubated at 37 °C for 24 hours. Subsequently, the bacterial colony was inoculated in 5 mL of nutrient broth (NB) tube and incubated for 24 hours at 37 °C. The optical density of bacterial culture was diluted with 0.85% normal saline to achieve a 0.5 McFarland standard or an absorbance of 0.08–0.13 at 625 nm (OD_{625}) using a

spectrophotometer (Clinical and Laboratory Standards Institute, 2012). A sterile cotton swab was dipped in the bacterial solution and then swabbed onto the surface of a PDA plate. A 5 mm diameter mycelial plug from a three-day old fungal isolate grown on PDA medium was transferred to the surface of the PDA plate, which had been previously coated with bacteria. Negative and positive controls were prepared using water agar (WA) medium and a fungal plug on the PDA Petri dish, respectively. Each assay plate consisted of four replicates of agar plugs. The co-culture plate was incubated at room temperature for 24–72 hours. The antibacterial activity was determined by measuring the diameter (mm) of the inhibition zone (Mathan et al., 2013). Fungal isolates exhibiting positive results were selected for secondary screening, involving the extraction of antimicrobial compounds and subsequent testing of their antibacterial activities.

Crude extraction from fungi

The fungal isolates were cultured in potato dextrose broth (PDB) and incubated at room temperature (30±2 °C) for a period of 21 days. The mycelium was separated from the fermented broth by filtering through Whatman No. 1 filter paper. Subsequently, both parts were extracted with equal volumes of ethyl acetate (EtOAc). The samples were vigorously shaken in a rotatory shaker for a duration of 5–7 days. The solvent phase from both parts was collected and subjected to evaporation using rotary vacuum evaporators (40 °C, 150–240 mbar). The fungal crude extract was maintained in a desiccator until required for further studies (Figure 1).

Figure-1. Crude extraction of fungus with ethyl acetate (EtOAc). [Created in BioRender.com]

Testing antibacterial activity of crude extract by agar disk diffusion method

The agar disk diffusion method used to assess antibacterial activity was slightly modified based on Clinical and Laboratory Standards Institute (CLSI) (2012) and Phonmakham et al. (2018). To prepare the samples, the crude extract, consisting of mycelium and fermented broth, was dissolved in a mixture of dimethyl sulfoxide (DMSO) and ethanol in a 1:1 ratio. A stock solution with a concentration of 200 mg/mL was then serially diluted by a factor of two to obtain extract concentrations ranging from 100 mg/mL to 6.25 mg/mL. These dilutions were used to test the extract against six human pathogenic bacteria, following the same procedure as in the previous experiment. The bacterial strains were prepared and swabbed onto Mueller-Hinton agar (MHA) plates. As controls, gentamicin (100 µg/mL) was used as a positive control, while a mixture of DMSO and ethanol in a 1:1 ratio served as the negative control. The experiments were conducted in triplicate and incubated at 37 °C for 24 hours. The zones of antibacterial activity were then measured as the diameters of the inhibition zones and recorded in millimeters, as described by Mathan et al. (2013).

Screening for primary enzyme activities

Pre-inoculum preparation

The isolates were inoculated on PDA plates and placed in an incubator set at room temperature for a duration of 3 days. Fungal mycelial plugs, measuring 5 mm in diameter, were then transferred to a 120 mL tissue-culture bottle containing 10 mL of PDB medium. These bottles were subsequently incubated at room temperature until visible growth was observed, which occurred at both the 2-week and 4-week marks. To assess enzyme activity, 20 µl of supernatant from the bottle was utilized in accordance with the modified agar well diffusion method (Balouiri et al., 2016). Wells with a diameter of 5 mm were created in solidified agar, and all isolates were tested using a triple Petri dish, with each method employing three wells. The resulting clearing zone surrounding the wells was measured in millimeters and recorded (Gesheva and Vasileva-Tonkova, 2012).

Cellulase activities

To determine the cellulose activity of fungal isolates, the modified methods of Kasana et al. (2008) and Neethu et al. (2012) were used as follows: carboxymethylcellulose agar (CMCA) medium containing 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.2% Peptone, 0.2% CMC, and 1.7% agar with each fungal isolate's supernatant (20 µL) was carefully added to the wells of the CMCA medium. The positive reaction is 10 µL of 10 mg/mL cellulase in 0.05M sodium citrate buffer

(pH 4.8). Following this, all Petri dishes were incubated in darkness at room temperature overnight. To visualize the CMCA plates, they were flooded with 5 mL of Gram's iodine stain (1% KI and 0.5% I_2 in 300 mL distilled water) for 5 minutes. Subsequently, the surface was rinsed with distilled water. The hydrolysis of cellulose or CMC was indicated by the development of a clear zone around the wells, which was then measured in millimeters.

Pectinase activities

To assess the pectinase activity of fungal isolates, a modified method based on Sunitha et al. (2013) was used as follows: 20 µl of supernatants from each isolate were placed in the well of a pectin agar medium containing 0.5% pectin, 0.1% yeast extract, and 1.5% agar at pH 5. The positive control consisted of 10 µL of 10 mg/mL pectinase in pectinase buffer (0.1 M Sodium acetate, 5 mM Ethylenediaminetetraacetic acid (EDTA) at pH 4.5). Following this, all Petri dishes were left to incubate in darkness at room temperature overnight. Subsequently, the Petri dishes were flooded with 5 mL of 1% Cetyltrimethylammonium bromide (CTAB) staining solution for 10 minutes, after which the surface was rinsed with distilled water. The presence of a clear zone of hydrolysis around the wells (measured in millimeters) indicated the activity of pectinase.

Laccase activities

To assess the laccase activity of fungal isolates, the modified method described by Sunitha et al. (2013) was used as follows: 20 µl of the supernatants from each isolate were added to the well of glucose yeast extract peptone agar (GYPA) medium containing with 0.005 % 1-Napthol. The positive control consisted of 10 µl of 10 mg/mL of laccase in 0.1 M sodium citrate buffer (pH 4.5). Following this, all Petri dishes were incubated in darkness at room temperature overnight. The presence of purple coloration around the wells (in millimeters) served as an indicator of laccase activity.

Statistical analysis

Minitab software (version 19.2020.1) was used for statistical analysis, wherein one-way analysis of variance (ANOVA) was conducted and performed a Tukey test analysis ($p < 0.05$).

Results

Isolation and identification of coprophilous fungi

Twenty-seven distinguishable heat-resistant fungi were isolated from fresh cow dung, and the fungi were identified up to the genera level using morpho-molecular techniques (Table 1, Figure 2). The three fungal genera with the highest numbers of isolates were *Penicillium* (8 isolates), *Talaromyces* (7 isolates), and *Aspergillus* (5 isolates) as shown in Figures 2–7. According to Index Fungorum (7 November 2023), certain fungal species retain species for the current name as follows: *Pa. formosus* = *Pa. maximus*, *Pe. brefeldianum* = *Pe. dodgei*, and *Pe. janthinellum* or *Pe. paraherquei* = *Pe. simplicissimum*. The identification of taxa using the ITS via BLAST was effective in determining the isolates at the species level, including *As. terreus*, *Pe. javanicum*, *Pe. lineolatum*, and *T. trachyspermus*, as listed in Table 1. In the PDA medium, some fungal isolates exhibited solely sexual morph, such as *Aspergillus* sp. NTD-NG2-05. Morphologically, the isolate displayed ascomata, asci, and ascospores (Figure 5). Three isolates (NTD-NG2-30, NTD-SP3-11, and NTD-SP5-34) could not be identified through ITS even at the genus level due to the presence of only mycelium on PDA (Figure 8). *Pa. maximus* demonstrated a quicker colony growth compared to NTD-SP5-08 (85 mm), NTD-NG2-21 (78 mm), and NTD-NG1-02 (72 mm), respectively, along with sporulation occurring after 4 days on PDA medium. On the other hand, *Aspergillus* sp. NTD-SP4-32 exhibited a colony diameter of only 11 colony diameter (Figure 9).

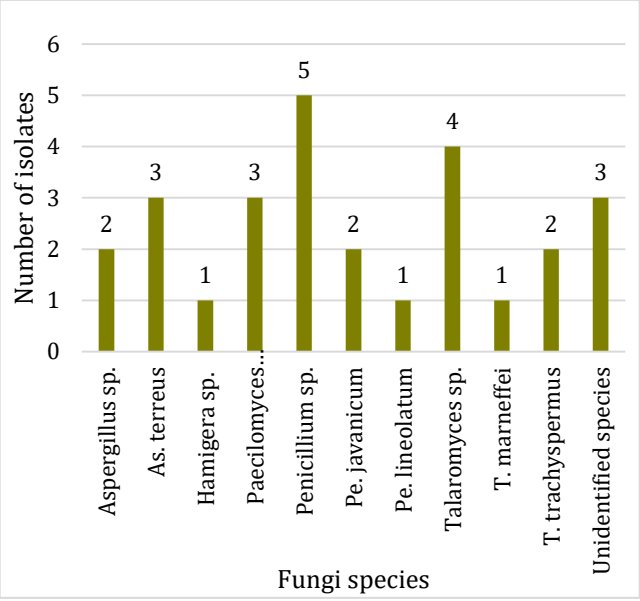

Figure-2. The number of species of coprophilous fungi isolated from cow dung

Table-1. The coprophilous fungi from cow dung and the search results from the BLAST analysis of the ITS region

Collection site	Isolates	Species recognized on BLAST	GenBank Accessions	Best hit	Query cover (%)	Identity (%)
Organic farm						
Rukkaset farm	NTD-NG1-02	*Paecilomyces maximus*	OP369047	KX134678 *Paecilomyces* sp.	100	99.49
				MN421908 *Pa. formosus***	100	99.32
				MN421872 *Pa. formosus***	100	99.32
				MN421864 *Pa. formosus***	100	99.32
	NTD-NG1-05	*Aspergillus terreus*	OP369048	MT530257 *As. terreus*	100	100
				MT530253 *As. terreus*	100	100
				MT530239 *As. terreus*	100	100
	NTD-NG1-09	*Talaromyces trachyspermus*	OP369049	MT528783 *T. trachyspermus*	100	100
				MK355723 *Paecilomyces* sp.	100	100
				GQ365160 *T. trachyspermus*	100	100
	NTD-NG2-04	*Penicillium javanicum*	OP369050	MK450698 *Pe. javanicum*	100	100
				MK775949 *Penicillium* sp.	100	100
				MH864718 *Pe. javanicum*	100	100
	NTD-NG2-05	*Aspergillus* sp.	OP369051	MK111645 *As. thermomutatus*	100	100
				MH859985 *As. Spinosus*	100	100
				OW984039 *As. thermomutatus*	100	100
	NTD-NG2-14	*Pe. lineolatum*	OP369052	MK450701 *Pe. lineolatum*	100	100
				MH861047 *Pe. lineolatum*	100	100
				NR_111500 *Pe. lineolatum*	96	100
	NTD-NG2-21	*Pa. maximus*	OP369053	MF780707 *Paecilomyces* sp.	100	99.83
				ON853875 *Pa. maximus*	100	99.83
				ON853846 *Pa. maximus*	100	99.83
	NTD-NG2-30	Unidentified species*	OP369054	HG996125 uncultured Pleosporales	96	99.4
				LR993965 uncultured fungus	96	99
				HG996320 uncultured Pleosporales	96	98.8
	NTD-NG2-39	*Hamigera* sp.	OP369055	MH865517 *H. avellanea*	100	99.82
				JQ796873 *H. avellanea*	100	99.65
				NR_137734 *H. fusca*	99	99.65
				GU092938 *H. fusca*	99	99.65
My Land New	NTD-SP4-32	*Aspergillus* sp.	OP369065	MT529916 *As. proliferans*	100	100
Theory Agriculture				MT316337 *As. chevalieri*	100	100
Learning Center				MT487826 *As. montevidensis*	100	100
	NTD-SP4-50	*As. terreus*	OP369066	MT530257 *As. terreus*	100	100
				MT530253 *As. terreus*	100	100
				MT530239 *As. terreus*	100	100
Field-fed cows	NTD-SP1-01	*As. terreus*	OP369056	MT530257 *As. terreus*	100	100
				MT530253 *As. terreus*	100	100

				MT530239 *As. terreus*	100	100
	NTD-SP1-03	*T. marneffei*	OP369057	MT530070 *Eurotiales* sp.	100	100
				MN856261 *T. marneffei*	100	100
				MN856253 *T. marneffei*	100	100
	NTD-SP2-01	*Pe. javanicum*	OP369058	MK450698 *Pe. javanicum*	100	100
				MK775949 *Penicillium* sp.	100	100
				MH864718 *Pe. javanicum*	100	100
	NTD-SP2-16	*Penicillium* sp.	OP369059	MK450735 *Penicillium* sp.	100	99.82
				MH858155 *Pe. brefeldianum***	100	99.82
				KM268710 *Pe. janthinellum***	100	99.82
	NTD-SP2-49	*Talaromyces* sp.	OP369060	MK072976 *Talaromyces* sp.	99	98.41
				JN899391 *Talaromyces* sp.	99	98.04
				HQ607791 *T. verruculosus*	100	97.16
	NTD-SP2-59	*Penicillium* sp.	OP369061	MT543120 *Penicillium* sp.	100	99.82
				MH860152 *Pe. senticosum*	100	99.82
				MH859859 *Pe. parvum*	100	99.82
				KM023348 *Pe. paraherquei***	100	99.82
	NTD-SP3-02	*Penicillium* sp.	OP369062	MK450735 *Penicillium* sp.	100	99.82
				MH858155 *Pe. brefeldianum***	100	99.82
				KM268710 *Pe. janthinellum***	100	99.82
	NTD-SP3-07	*Penicillium* sp.	OP369063	MK450735 *Penicillium* sp.	100	99.82
				MH858155 *Pe. brefeldianum***	100	99.82
				KM268710 *Pe. janthinellum***	100	99.82
	NTD-SP3-11	Unidentified species*	OP369064	MN306059 *Sarocladiumterricola*	100	100
				KJ524675 *Hirsutella* sp.	100	100
				KM051400 *Eutypa* sp.	100	100
	NTD-SP5-08	*Pa. maximus*	OP369067	ON853869 *Pa. maximus*	100	99.49
				MH859718 *Pa. maximus*	100	99.15
				FJ389921 *Pa. formosus***	96	99.82
	NTD-SP5-12	*Penicillium* sp.	OP369068	MH865344 *Pe. daleae*	100	99.65
				MK450722 *Pe. longicatenatum*	100	99.47
				MH858960 *Pe. abidjanum*	100	99.47
	NTD-SP5-34	Unidentified species*	OP369069	MW194291 *Allocanariomyces americanus*	100	99.26
				KU869524 *Chaetomium* sp.	99	99.26
				KP870086 *Colletotrichum gloeosporioides*	100	99.08
	NTD-SP5-43	*Talaromyces* sp.	OP369070	HQ607791 *T. verruculosus*	100	98.89
				MH865566 *T. aculeatus*	100	98.71
				MZ045695 *T. haitouensis*	100	98.71
	NTD-SP5-46	*Talaromyces* sp.	OP369071	MT530163 *T. oumae-annae*	100	100
				MT463516 *T. liani*	100	100
				MN864269 *T. brevis*	100	100
				MH856409 *T. flavus* var. *flavus*	100	100

				MG780394 *T. pinophilus*	100	100
	NTD-SP5-48	*Talaromyces* sp.	OP369072	MK450745 *Talaromyces* sp.	100	99.28
				MK450744 *Talaromyces* sp.	100	99.1
				KT216044 *T. pinophilus*	95	99.82
	NTD-SP5-52	*T. trachyspermus*	OP369073	MT528783 *T. trachyspermus*	100	100
				MK355723 *Paecilomyces* sp.	100	100
				GQ365160 *T. trachyspermus*	100	100

*The identification of fungi at the species level using the ITS region was not possible.
** According to Index Fungorum, certain fungal species still maintain a different species name for their current identification.

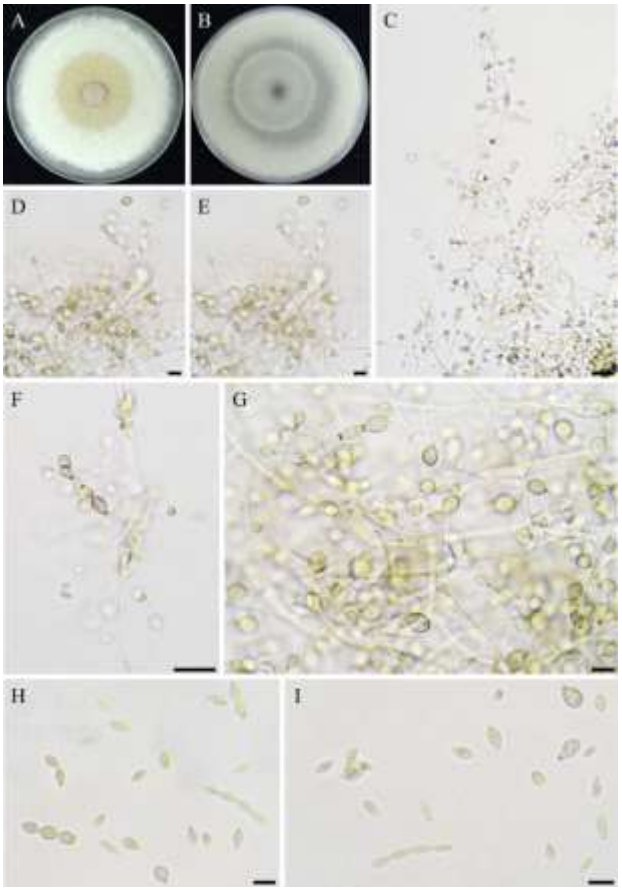

Figure-3. *Paecilomyces maximus* isolate NTD-NG1-02. A-B) Colonies on PDA, C-G) Conidiophores with conidial structures, H-I) Conidia. Scale bars = 10 µm.

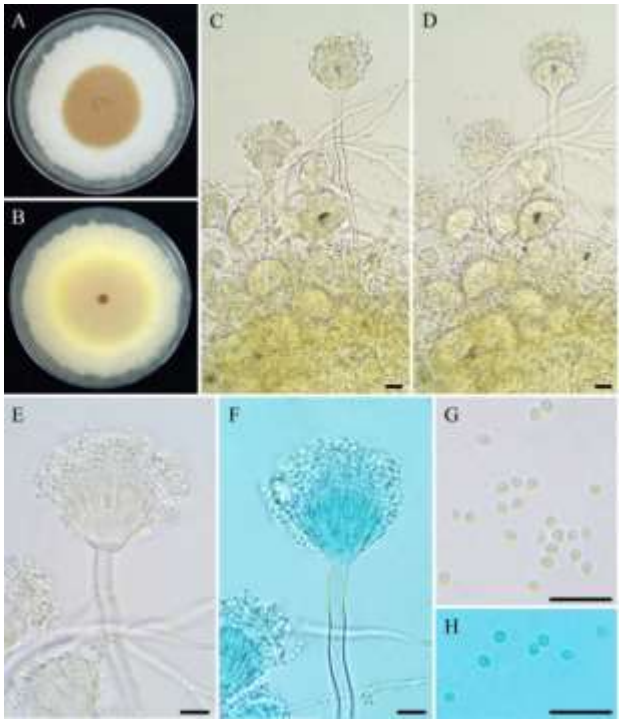

Figure-4. *Aspergillus terreus* isolate NTD-NG1-05. A-B) Colonies on PDA, C-F) Conidiophores with conidial heads, G-H) Conidia. Scale bars = 10 µm.

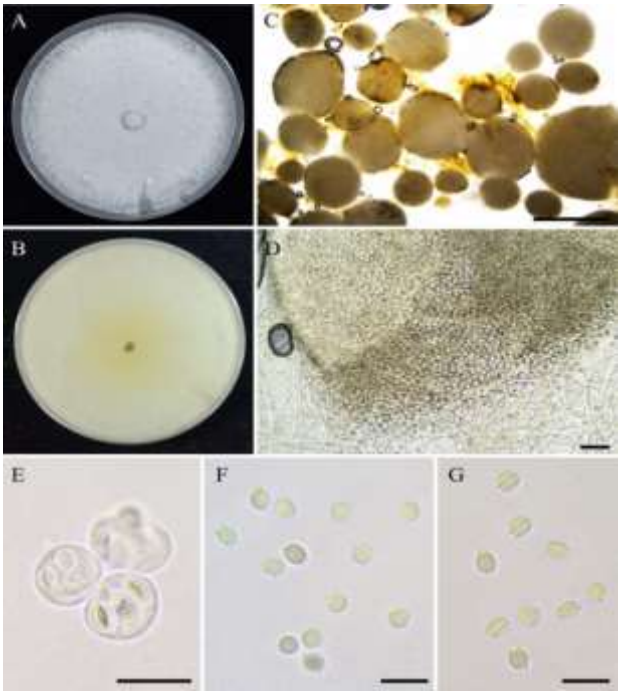

Figure-5. ***Aspergillus*** **sp. isolate NTD-NG2-05. A-B) Colonies on PDA, C-D) Ascomata, E) Asci., F-G) Ascospores. Scale bars = C-D = 20 µm and E = 10 µm.**

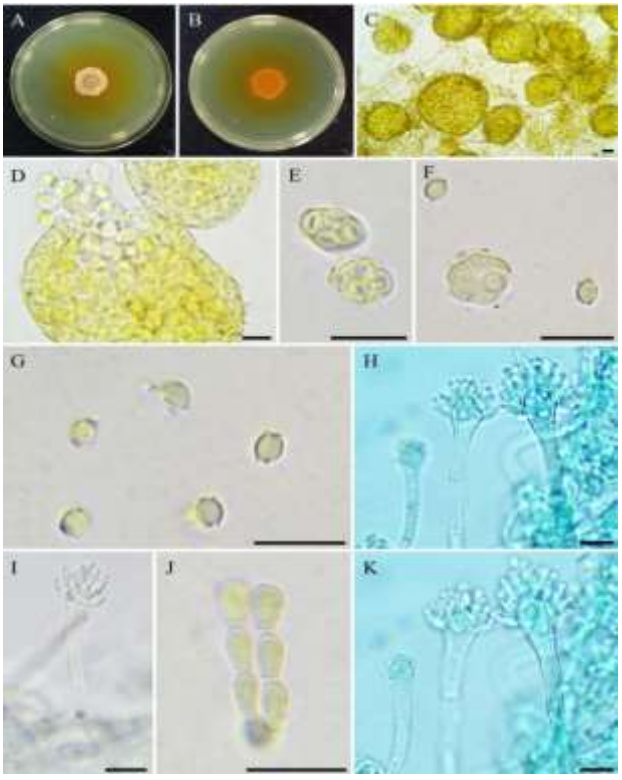

Figure-6. ***Aspergillus*** **sp. isolate NTD-SP4-32. A-B) Colonies on PDA, C-D) Ascomata, E) Asci., F) Asci and Ascospores, G) Ascospores. H-I, K) Conidiophores, J) Conidia. Scale bars = C-K = 10 µm.**

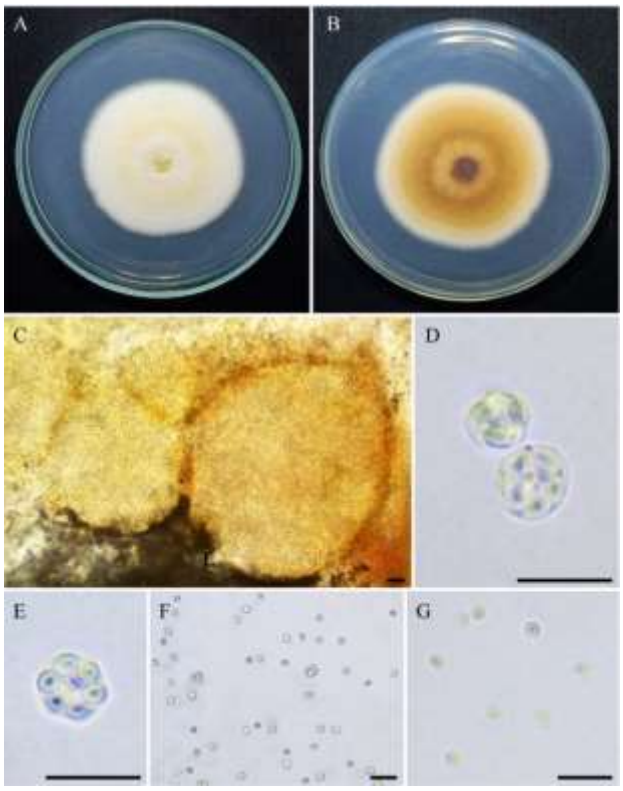

Figure-7. ***Talaromyces*** **sp. isolate NTD-SP5-48. A-B) Colonies on PDA, C) Ascomata, D-E) Asci., F) Ascospores. Scale bars = 10 µm.**

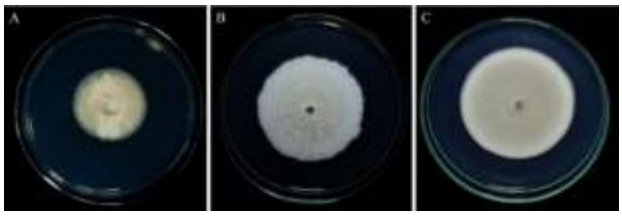

Figure-8. Colonies of unidentified fungal isolates on PDA medium. A) Isolates NTD-NG2-30, B) Isolates NTD-SP3-11, and C) Isolates NTD-SP5-34.

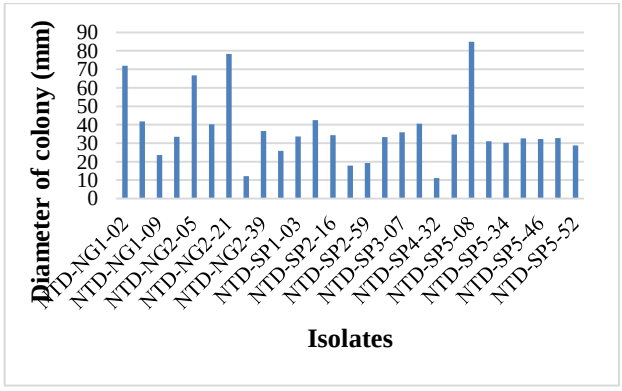

Figure-9. Colony diameter of coprophilous fungal isolates on PDA medium at 4 days.

Antibacterial activity

Primary antibacterial activity screening by agar plug diffusion method

The presented results demonstrated varying degrees of inhibition, indicated by the formation of clear zones, against six bacterial strains (Table 2). Among the isolates tested, *Pe. javanicum* NTD-SP2-01 and *Talaromyces* sp. NTD-SP5-48 exhibited inhibition against all the bacterial strains, with *Talaromyces* sp. NTD-SP5-48 showing the highest efficacy against both Gram-negative bacteria. *As. terreus* NTD-NG1-05 displayed the best inhibition efficiency against five bacterial strains, except for *Ps. aeruginosa* (Figure 10). Consequently, *As. terreus* NTD-NG1-05 and *Talaromyces* sp. NTD-SP5-48 were chosen for further screening using the ethyl acetate extract method. Among the *Pa. maximus* isolates, only *Pa. maximus* NTD-SP5-08 demonstrated inhibition efficiency against *Ps. aeruginosa*. On the other hand, *Pa. maximus* NTD-NG1-02 and NTD-NG2-21, *Pe. lineolatum* NTD-NG2-14, *Penicillium* sp. NTD-SP5-12, and an unidentified species NTD-NG2-30 did not exhibit any inhibition activity against the tested bacterial strains.

The antibacterial activity of crude extract by agar disk diffusion method

The ethyl acetate extracts were evaluated for their antibacterial properties using the agar disk diffusion method (Table 3). An effective antibacterial activity was observed in the range of 6.25–100 mg/mL against 5 out of 6 bacterial strains. The fermented broth (FB) exhibited higher inhibition efficiency compared to the mycelial (MY) extraction. Both isolates primarily targeted Gram-positive bacteria. The FB extract from both isolates displayed significant antibacterial activity against *B. subtilis* at 0.125 mg/disk (12.5 mg/mL). Whereas, *As. terreus* NTD-NG1-05 only inhibited *St. epidermidis* (Figure 11). Among Gram-negative bacteria, only the ME extract of *Talaromyces* sp. NTD-SP5-48 showed antibacterial activity against *Ps. aeruginosa* at 1 mg/disk (100 mg/mL).

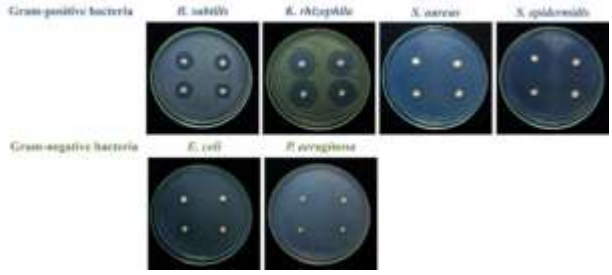

Figure-10. Agar plug diffusion method of *Aspergillus terreus* isolate NTD-NG1-05 against six human pathogenic bacteria (*Bacillus subtilis, Kocuria rhizophila, Staphylococcus aureus, St. epidermidis, Escherichia coli* and *Pseudomonas aeruginosa*).

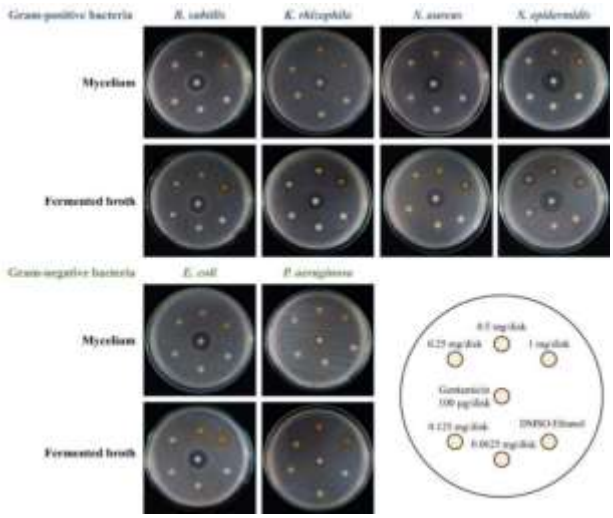

Figure-11. Agar disk diffusion method of *Aspergillus terreus* isolate NTD-NG1-05 from ethyl acetate extract (Mycelium and fermented broth) against six human pathogenic bacteria (*Bacillus subtilis, Kocuria rhizophila, Staphylococcus aureus, St. epidermidis, Escherichia coli* and *Pseudomonas aeruginosa*).

Table-2 The agar plug diffusion method of fungal isolates against six bacteria

Species recognized on BLAST	Isolates	Diameter of clear zone in mm (mean±SD, n=4)					
		Gram-positive bacteria				Gram-negative bacteria	
		B. subtilis	*K. rhizophila*	*St. aureus*	*St. epidermidis*	*E. coli*	*P. aeroginosa*
Aspergillus sp.	NTD-NG2-05	-	9.69±0.36^{GH}	-	12.79±0.34^{EFG}	-	-
	NTD-SP4-32	-	7.71±0.21^{I}	7.31±0.39^{E}	8.13±0.17^{M}	-	-
As. terreus	NTD-NG1-05	19.28±0.39^{A}	26.46±0.84^{A}	27.46±1.49^{A}	32.52±0.63^{A}	10.00±0.49^{A}	-
	NTD-SP1-01	12.75±0.35^{E}	24.23±1.21^{B}	10.90±0.63^{C}	23.43±0.79^{B}	6.53±0.07^{D}	-
	NTD-SP4-50	8.42±0.08^{GH}	18.67±0.41^{D}	-	10.99±1.15^{HI}	-	-
Hamigera sp.	NTD-NG2-39	18.11±0.44^{B}	21.01±0.69^{C}	-	-	-	-
Paecilomyces maximus	NTD-NG1-02	-	-	-	-	-	-
	NTD-NG2-21	-	-	-	-	-	-
	NTD-SP5-08	-	-	-	-	-	11.79±0.20^{A}
Penicillium sp.	NTD-SP2-16	14.64±0.46^{D}	16.37±0.62^{E}	-	10.00±0.43^{IJK}	-	8.29±0.57^{D}
	NTD-SP2-59	-	-	-	8.54±0.58^{LM}	-	-
	NTD-SP3-02	-	9.01±1.29^{HI}	-	8.03±0.37^{M}	-	-
	NTD-SP3-07	8.68±0.67^{G}	15.84±0.27^{E}	8.63±0.26^{D}	16.40±0.15^{D}	8.54±0.13^{B}	-
	NTD-SP5-12	-	-	-	-	-	-
Pe. javanicum	NTD-NG2-04	9.64±0.23^{F}	12.26±0.75^{F}	-	11.68±0.22^{GH}	-	-
	NTD-SP2-01	7.82±0.15^{HI}	9.36±0.58^{H}	10.42±0.17^{C}	13.70±0.18^{E}	8.20±0.28^{C}	9.05±0.28^{CD}
Pe. lineolatum	NTD-NG2-14	-	-	-	-	-	-
Talaromyces sp.	NTD-SP2-49	7.55±0.01^{IJ}	11.31±0.41^{F}	-	-	-	9.56±0.21^{BC}
	NTD-SP5-43	8.06±0.06^{HI}	22.22±1.11^{C}	-	12.81±0.65^{EF}	-	-
	NTD-SP5-46	7.09±0.09^{J}	19.11±0.26^{D}	10.71±0.02^{C}	11.57±0.23^{H}	-	-
	NTD-SP5-48	10.06±0.19^{F}	12.06±0.26^{F}	8.41±0.21^{D}	10.72±0.23^{HIJ}	9.79±0.30^{A}	10.36±0.29^{B}
T. marneffei	NTD-SP1-03	6.44±0.17^{K}	12.57±0.28^{F}	8.27±0.20^{D}	8.98±0.22^{KLM}	-	-
T. trachyspermus	NTD-NG1-09	8.24±0.25^{GH}	11.81±0.78^{F}	-	11.71±0.63^{FGH}	-	10.05±2.27^{BC}
	NTD-SP5-52	-	9.79±0.31^{GH}	-	9.63±0.48^{JKL}	-	-
Unidentified species	NTD-NG2-30	-	-	-	-	-	-
	NTD-SP3-11	17.19±0.05^{C}	12.28±0.28^{F}	17.76±0.59^{B}	20.18±0.50^{C}	-	-
	NTD-SP5-34	-	11.18±0.33^{FG}	-	-	-	-

Significance level, $p < 0.05$.; Clear zone diameter index: – no clear zone.

Table-3. The agar disk diffusion method of fungal isolates ethyl acetate extracts against six bacteria

Fungi	Part*	Concentration (mg/disk)	Diameter of clear zone in mm (mean±SD, n=6)					
			Gram-positive bacteria				Gram-negative bacteria	
			B. subtilis	*K. rhizophila*	*St. aureus*	*St. epidermidis*	*E. coli*	*P. aeroginosa*
Aspergillus terreus NTD-NG1-05	MY	0.0625	-	-	-	-	-	-
		0.125	-	-	-	-	-	-
		0.25	-	-	-	-	-	-
		0.5	6.87±0.23	-	-	-	-	-
		1	8.10±0.54	-	6.68±0.33	7.37±0.12	-	-
		Gentamycin**	18.43±0.72	15.62±0.37	16.67±0.85	20.88±0.64	17.77±0.14	8.50±0.24
	FB	0.0625	-	-	-	-	-	-
		0.125	6.85±0.30	-	-	7.83±0.77	-	-
		0.25	7.40±0.33	8.02±0.39	7.80±0.51	10.03±0.97	-	-

		0.5	7.05±0.54	7.88±0.48	8.00±0.34	10.00±0.35	-	-
		1	8.28±0.37	9.38±0.50	9.33±0.31	12.00±1.19	-	-
		Gentamycin	18.35±0.50	15.85±0.85	18.98±0.60	21.00±1.07	17.33±1.18	8.38±0.37
Talaromyces sp. NTD-SP5-48	MY	0.0625	-	-	-	-	-	-
		0.125	-	-	-	-	-	-
		0.25	-	-	-	-	-	-
		0.5	-	-	-	-	-	-
		1	6.53±0.30	-	-	-	-	-
		Gentamycin	18.43±0.41	9.90±0.73	17.43±0.64	18.85±0.87	16.75±0.51	8.33±0.45
	FB	0.0625	-	-	-	-	-	-
		0.125	6.48±0.10	-	-	-	-	-
		0.25	7.53±0.62	-	6.60±0.06	-	-	-
		0.5	9.02±0.34	7.27±0.52	7.25±0.19	6.83±0.31	-	-
		1	10.93±0.85	8.72±0.45	8.22±0.21	8.32±0.37	-	6.25±0.08
		Gentamycin	19.35±1.20	16.05±0.72	18.52±0.36	19.38±0.66	16.20±2.41	8.27±0.45

*Part of extracts: mycelium (MY), fermented broth (FB); **Concentration of gentamycin = 100 µg/disk; Significance level, $p < 0.05$.
Clear zone diameter index: – no clear zone

Table-4. Enzyme activity of fungal isolates

Species recognized on BLAST	Isolates	Cellulase		Pectinase	Laccase
		2 weeks	4 weeks	2 and 4 weeks	2 and 4 weeks
Aspergillus sp.	NTD-NG2-05	+++	+++	-	-
	NTD-SP4-32	-	-	-	-
As. terreus	NTD-NG1-05	+++	+++	-	-
	NTD-SP1-01	+++	++++	-	-
	NTD-SP4-50	+++	++++	-	-
Hamigera sp.	NTD-NG2-39	++	+++	-	-
Paecilomyces maximus	NTD-NG1-02	+++	+++	-	-
	NTD-NG2-21	+++	++++	-	-
	NTD-SP5-08	+	+++	-	-
Penicillium sp.	NTD-SP2-16	+	-	-	-
	NTD-SP2-59	+++	++++	-	-
	NTD-SP3-02	-	+++	-	-
	NTD-SP3-07	+++	+++	-	-
	NTD-SP5-12	++	+++	-	-
Pe. javanicum	NTD-NG2-04	++++	+++	-	-
	NTD-SP2-01	+++	+	-	-
Pe. lineolatum	NTD-NG2-14	+	++	-	-
Talaromyces sp.	NTD-SP2-49	++++	++++	-	-
	NTD-SP5-43	+++	+++	-	-
	NTD-SP5-46	++++	++++	-	-
	NTD-SP5-48	++++	++++	-	-
T. marneffei	NTD-SP1-03	++	+++	-	-
T. trachyspermus	NTD-NG1-09	+++	++++	-	-
	NTD-SP5-52	+++	++++	-	-
Unidentified species	NTD-NG2-30	-	++++	-	-
	NTD-SP3-11	++	+++	-	-
	NTD-SP5-34	+	+++	-	+*

Symbols: –, negative result: no clear zone; +, positive result: a clear zone or colour: +, zone of 1–2 mm; ++, zone of 3–5 mm; +++, zone of 5 mm, and ++++ zone of 10 mm and more.
+*, purple colour

Screening for enzyme activity

Cellulase activities

The cellulase activity of the fungal isolates was assessed against CMC, with almost all isolates (26 isolates) exhibiting this activity, as indicated by the clear zone observed (Figure 12). The cellulase activity of most isolates showed an increase from 2 to 4 weeks, except *Pe. javanicum* NTD-SP2-01 and *Penicillium* sp. NTD-SP2-16, which displayed lower cellulase activity. In contrast, *Aspergillus* sp. NTD-SP4-32 did not show any cellulase activity and exhibited no enzyme activity at all (Table 4). Among the genera studied, *Talaromyces* demonstrated the highest cellulase activity. The top four isolates in terms of cellulase enzyme activity were *T. trachyspermus* NTD-NG1-09 (14 mm), *Talaromyces* sp. NTD-SP2-49 (13 mm), *Talaromyces* sp. NTD-SP5-46 (13 mm), and *Talaromyces* sp. NTD-SP5-48 (13 mm).

Pectinase and laccase activities

Among the 27 fungal isolates examined, none exhibited pectinase and laccase activity, except the unidentified fungal isolate NTD-SP5-34, which showed a laccase activity as indicated by a purple colour (Figure 12, Table 4).

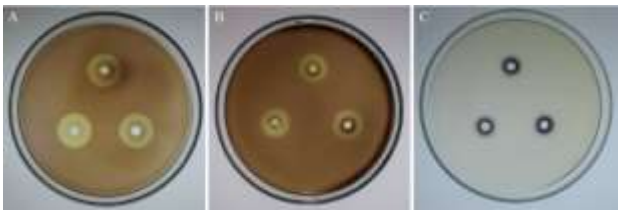

Figure-12. Enzyme activity on Petri dish. **A-B) Cellulase activity with Gram's iodine solution, *Talaromyces trachyspermus* NTD-NG1-09:** (**A**) **and *Penicillium javanicum* NTD-NG2-04** (**B**), **C) Laccase activity with the purple zone of 1-naphthol around colonies**.

Discussion

The findings presented in this study demonstrate that the heat-resistant coprophilous fungi, viz. species of *Aspergillus*, *Penicillium* and *Paecillomyces*, that was similar to Piecková et al. (2019), were identified in cow dung samples collected in Thailand. These fungi are classified under the phylum Ascomycota, which aligns with results reported in previous studies conducted in India (Thilagam et al., 2015). Additionally, these fungi are commonly found in compost soils (Witfeld et al., 2021), and have been reported to contaminate various food and feed products (Houbraken et al., 2008; Sheikh-Ali et al., 2014; Garnier et al., 2017; Berni et al., 2017). Certain species of *Aspergillus* have been reported to cause infections in both humans and animals (Paulussen et al., 2017). The ITS region serves as the primary and universal barcode for the identification of fungi. It exhibits a significant degree of variability among fungi across different taxonomic levels, enabling successful PCR amplification and superior identification for a wide range of fungal species (Schoch et al., 2012; Raja et al., 2017; Lücking et al., 2020). Although the ITS barcode may not provide species level identification for all fungi, it remains sufficiently accurate for most genera. To enhance the classification of fungal ITS barcodes at the species level, a bioinformatics tool called Its2vec has been developed (Wang et al., 2020). In subsequent research, the secondary barcoding markers namely β-tubulin II (TUB2), RNA polymerase II largest (RPB1) and second largest (RPB2) subunits, and translational elongation factor 1α (TEF1α) are being increasingly utilized in the identification of fungi when the ITS region fails to provide accurate identification. In light of the One Fungus-One Name (1F1N) initiative, it was discovered that many species originated from recombination events. Consequently, database containing taxonomic and nomenclatural species names can be accessed via Index Fungorum (https://www.indexfungorum.org/Names/Names.asp), MycoBank (https://www.mycobank.org/), and the National Library of Medicine (https://www.ncbi.nlm.nih.gov).

Neosartorya species, now classified as *Aspergillus*, have been used in the synthesis of bioactive compounds (de Sá et al., 2022). According to Jawaid et al. (2019), an isolate of *As. terreus* MK-1 demonstrated the ability to produce antimicrobial compounds, as evidenced by the crude ethyl acetate extract's activity against *Ps. aeruginosa*. While the *Aspergillus* isolates did not exhibit activity against *Ps. aeruginosa*, they displayed significant antibacterial activity against various bacterial strains such as *B. subtilis*, *E. coli*, *K. rhizophila*, *St. aureus*, and *St. epidermidis*. *Talaromyces* spp. have also shown positive antibacterial activity, consistent with the findings of Song et al. (2022).

Furthermore, Zhai et al. (2016) identified 221 bioactive compounds from *Talaromyces* sp. (including *Penicillium* sp.), encompassing alkaloids, esters, peptides, polyketides, quinones, steroids, terpenoids, and other compound classes.
Several species, including *Aspergillus*, *Fusarium*, *Hamigera*, *Paecilomyces*, *Talaromyces*, and *Trichoderma* have been identified as efficient producers of cellulase and other enzymes (Sunitha et al., 2013; Elsababty et al., 2015; El-Gendi et al., 2022; Faheina Junior et al., 2022), which aligns with the results obtained in this study that most isolates exhibited the potential to produce cellulase. None of the isolates examined in this study exhibited pectinase activity, in contrast to the findings reported by Elsababty et al. (2015) and Haile and Ayele (2022) regarding fungi such as *As. niger*, *As. awamori*, *Pe. restrictum*, *Tr. viride*, *M. piriformis*, and *Yarrowia lipolytica*. Nevertheless, it is crucial to continue investigating the optimal conditions for fungal growth and enzyme secretion, including factors such as temperature and growth medium.

Conclusion

In this study, several coprophilous fungi isolated from cow dung have exhibited potential for the synthesis of secondary metabolites and the enzymatic degradation of lignocellulose, with potential applications in the hydrolysis of pre-treated biomass. *As. terreus* NTD-NG1-05, *Pe. javanicum* NTD-SP2-0, and *Talaromyces* sp. NTD-SP5-48 have demonstrated efficiency in antibacterial applications. Most isolates, including *Aspergillus* spp., *Hamigera* sp., *Paecilomyces maximus*, *Penicillium* spp., and *Talaromyces* spp. displayed significant cellulose production capabilities within a 4-week period. The sole unidentified species isolate NTD-SP5-34 in this study exhibited a recognizable laccase activity.

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Source of Funding: None.

Contribution of Authors

Tangthirasunun N: Designed the methodology and conceptualized the research, provided oversight for the study and conducted a portion of the experiments, gathered and analyzed the data, drafted the original manuscript, reviewed and approved it.
Bhat DJ: Reviewed and revised the manuscript for accuracy and clarity.
Poeaim S: Assisted in supervising the research methodology and study, as well as reviewed and edited the manuscript.

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