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Bioprospecting of novel and biologically active compounds and enzymes from a new fungal biobank for industrial biotechnology

Jaswinder Kaur[1], Patrick Murray[1] and Catherine Collins[1*]

Abstract

Here, we present methods for screening any newly isolated microbial biobank for enzymes, antioxidants, and additional secondary metabolites. Bioactive compounds and enzymes produced by fungi have many applications in industry as valuable leads for pharmaceuticals, nutraceuticals, and potential strains for industrial biotechnology. In this study, fungi collected from Irish habitats were purified and identified by sequencing Internal Transcribed Spacer (ITS) ribosomal DNA regions. Reducing sugars released because of enzyme hydrolysis was used to estimate the relative enzyme activity for carboxymethyl cellulase, pectinase, *β*-xylanase, arabinoxylanase, mannanase, and galacto-mannanase in the fungal extracts and was normalized against the crude protein content to express relative enzyme activity/milligram. The best enzyme producers were as follows: F1 *Clonostachys rosea* demonstrated 351.63 U/mg *β*-xylanase and 645.50 U/mg arabinoxylanase activity; F2 *Penicillium expansum* had 25 U/mg cellulase and 112.5 U/mg pectinase activity; F3 *Fusarium avenaceum* had 139.36 U/mg arabinoxylanase, 52.33 U/mg galacto-mannanase, and 45 U/mg mannanase activity; and F9 *Trichoderma koningii* had 372.15 U/mg *β*-xylanase and 655.18 U/mg arabinoxylanase activity. In addition to enzyme activity, these strains also exhibited antioxidant activity when tested in Oxygen radical absorbance capacity (ORAC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric ion reducing antioxidant power (FRAP) assays, with activity expressed as µmol Trolox equivalents (TE) per gram of dried fungal extract. In results from the ORAC assay, F1 *C. rosea* reported 655,669 µmol TE/g; F2 *P. expansum* had 636,889 µmol TE/g; and F3 *F. avenaceum* had 4,488,035 µmol TE/g. High DPPH activity was observed for F1 *C. rosea* at 12,572 µmol TE/g, F3 *F. avenaceum* at 22,646 µmol TE/g, and *F9 T. koningii* at 22,558 µmol TE/g. F1 *C. rosea*, F3: *F. avenaceum*, and F9 *T. koningii* also reported high FRAP activity at 36,019 µmol TE/g, 69,325 µmol TE/g, and 25,812 µmol TE/g, respectively. In terms of secondary metabolites, the main compounds detected for the isolates were F1 *C. rosea* produced benzoic acid, 3-pyridinepropionic acid, and cyclo(-Gly-Phe); F2 *P. expansum* produced Cyclo(-Gly-Phe) and prenitremone A; F3 *F. avenaceum* produced cyclo(-Leu-Leu) and *p*-anisaldehyde; and F9 *T. koningii* produced cyclo(-Gly-Phe), canescin, citreoviridin X, and cyclopaldic acid. These selected isolates indicate that Irish fungi are a potential source of enzymes and novel biologically active compounds useful in industrial biotechnology and bioproduction supporting sustainability and responsible environmental management.

Keywords Fungal biotechnology, Enzymes, Antioxidant, Secondary metabolites, Fungal biobank

Introduction

Fungi are renowned for the production of enzymes, pigments, vitamins, lipids, and glycolipids at an industrial scale [1–3]. Fungal enzymes account for more than 50% of the total enzyme market with species of *Aspergillus*,

*Correspondence:
Catherine Collins
Catherine.Collins@tus.ie
[1] Shannon Applied Biotechnology Center, Technological University of the Shannon, Limerick V94 EC5T, Ireland

Trichoderma, *Rhizopus,* and *Penicillium* satisfying commercial-scale requirements [4]. Fungi are continuously explored for novel enzymes capable of meeting industrial requirements with improved efficiency. Fungal enzymes are advantageous in that they can be produced by culturing fungal enzyme producers on low-cost agricultural waste; the enzymes themselves can be purified with ease, are stable in harsh conditions, and have high catalytic capacity [4]. Given such attributes, fungal enzymes have applications in industries such as food and beverage [5], pulp, and paper [6], as well as textiles [7]. They play an important role in the biodegradation of lignin, decolorization of environmentally toxic dyes, and bioremediation of pollutants such as plastics [8, 9]. In addition to enzyme production, fungi are known to produce antioxidant, anticancer, and antimicrobial activities [1–12]. Secondary metabolites from fungi such as polyketides, flavonoids, terpenoids, phenolic acids, organic acids, and peptides demonstrate radical scavenging antioxidant ability. These compounds have applications in the food, nutraceutical, and pharmaceutical industry [13, 14]. For example, Kojic acid is used as an antioxidant in industry. Other fungal secondary metabolites of industrially relevant compounds include the antibiotics Penicillin G, Cephalosporin, Pleuromutilin, and Griseofulvin, and the drugs Cyclosporin A and Mycophenolic acid are used to prevent organ rejection [15].

To date, there has been no exploration of fungal populations from Irish habitats for useful compounds and enzymes that they could produce. There has been a study performed by O'Hanlon and Harrington where they examined the macrofungal communities of Irish native trees and reported 183 macrofungal species over a 3-year period from four forest types. This was a population study and did not report on any bioactivities produced by such macro fungi [16].

The aim of this study was to create a fungal biobank and examine the isolates collected from Irish habitats for enzyme, antioxidant activity, and secondary metabolite production. The methods highlighted in this study can act as a guide towards the assessment of future fungal biobanks targeting industrially relevant bioactivities and secondary metabolites. After isolating the fungi from Irish habitats and culturing them on plant-based substrates, the crude extracts were screened for six enzymatic activities, namely carboxymethyl cellulase (E.C.3.2.1.4), pectinase (E.C.4.2.2.10), β-xylanase, arabinoxylanase (E.C 3.2.1.8), mannanase, and galacto-mannanase (E.C.3.2.1.78), all of which play a key role in plant biomass degradation. In addition, the crude extracts of isolated fungal strains were screened for antioxidant activity using a combination of biochemical assays (ORAC, DPPH, and FRAP). Also, a screening method was developed to analyze the secondary metabolite profiles in the extracts of fungi using liquid chromatography coupled with mass spectrometry (LC–MS). Metabolites profiled included peptides, alkaloids, antibiotics, and antioxidants. The range and use of bioactivity assays presented here showcase how a fungal biobank can be generated and screened with a view towards exploring the isolated fungi for health, nutraceutical, enzymatic, and biotechnology applications.

Materials and methods

Chemicals

All chemicals used in this laboratory were of standard analytical grade. Ultrapure water from the TKA Genpure water system was used to prepare solutions. Ammonium acetate ultra was sourced from Fluka. Ethanol, methanol, and dichloromethane were purchased from Lennox Laboratory supplies. Wheat Arabinoxylan low viscosity ~ 7 cSt was provided by Megazyme. Wheat bran, oat bran, and sorghum were sourced from the local health food store. Potato dextrose agar; D-(+)-Mannose, Manganese(II) chloride tetrahydrate; acetic acid; 3,5-dinitrosalicylic acid; HEPES; cobalt(II) chloride (97%); sodium chloride Sigma ultra (99.5%); potassium phosphate monobasic (≥ 99.0%); sodium carbonate; 3,5-dinitrosalicylic acid; d-(+)-Mannose; Xylan (from beechwood); locust bean gum from *Ceratonia siliqua* seeds; Guar gum from *Cyamopsis tetragonoloba* seeds; corn steep liquor; albumin from bovine serum; Bradford reagent (98% electrophoresis); zinc sulfate heptahydrate; fluorescein, 2, 2'-Azobis (2-amidonopropane) dihydrochloride (AAPH); Trolox; sodium acetate; and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Aldrich.

Sample collection

Fungal samples were collected from woodlands and landscaped sites in Counties Clare and Limerick, Ireland. Mushrooms were also collected from the same sites from soil and plucked from decaying wood.

Isolating and purifying potential fungal isolates

Fungal growth conditions

Fungal samples collected from the wild were grown on potato dextrose agar (PDA) plates. Plates were incubated at 25 ℃ for 3–4 days until optimum growth stages were reached. Each fungal isolate was then inoculated onto a fresh PDA plate. This step was conducted until one pure culture from each fungal isolate was observed growing on the plate. Stocks of pure cultures have been prepared by taking 1 cm^2 plugs from the edge of an actively growing fungus on a PDA plate and immersing them in sterile 20% glycerol and storing them at minus 80 ℃ for

future research and are available upon request to other researchers.

DNA extraction and PCR of extracted DNA from fungal isolates

DNA from all the fungal isolates was extracted using the Gene JET Plant Genomic DNA purification kit (Thermo Scientific™) according to the manual. Mycelial cells from each isolate growing on a fresh PDA plate were ground into a fine powder using liquid nitrogen and used for DNA extraction. The extracted DNA was stored at − 20 ℃ until further use.

To identify the fungi, DNA was amplified with primers targeting the Internal Transcribed Spacer (ITS) region which has been used extensively in various studies with this region identified as having the highest probability for correct identification for the broadest group of sampled fungi [17]. PCR primers (ITS1 and ITS4) were based on published sequences targeting ITS regions [18]. PCR was conducted using Thermo scientific DreamTaq Green PCR master mix using TAQ polymerase acquired from ISIS™ DNA polymerase. DNA templates were either used neat or diluted (1 in 20 or 1 in 10). PCR reaction components consisted of 2 μL of DreamTaq buffer (10x), 0.2 μL of dNTPs (0.2 mM each), 1 μL of forward primer ITS 1 (5'TCCGTAGGTGAACCTGCGG3'), 1 μL of reverse primer ITS 4 (5'TCCTCCGCTTATTGATATGC3'), 13.6 μL of nuclease-free water, 0.2 μL of 5 U/μL Taq polymerase, and 2 μL of template DNA per reaction. Both forward and reverse primers were at a concentration of 10 pmol/μL. PCR was performed using the Applied Biosystems 2720 Thermo cycler under recommended thermal cycling conditions. Initial denaturation of 95 ℃ for 2 min was followed by 34 cycles of 95 ℃ for 30 s, 53 ℃ for 30 s (primer annealing), and 72 ℃ for 45 s (primer extension). A final extension of 72 ℃ for 10 min was incorporated followed by cooling to 4 ℃ until recovery of products. Once the reaction was complete the PCR products were stored at − 20 ℃ until further use. The DNA band for each PCR product was visualized on a 1.5% agarose gel stained with ethidium bromide. The gel was electrophoresed for 60 min at 120 V in 1 × TAE (tris–acetate-EDTA) buffer and visualized under UV light in a DNR imager.

Purification of PCR product and sequencing analysis of Internal Transcribed Spacer (ITS) region

Purification of PCR products to remove primers, nucleotides, polymerases, and salts was performed using the QIAquick PCR purification Kit (Qiagen) according to the kit's manual. The purified DNA was eluted in 50 μL 10 mM Tris–Cl, pH 8.5, and stored at − 20 ℃ until further use. The sequence reaction was conducted using the Sanger sequencing method at Source Bioscience (Waterford, Ireland). The forward primer, ITS1, was used to sequence the purified PCR product [18]. Basic Local Alignment Search Tool (BLAST) finds regions of local similarities between sequences [19]. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. For DNA sequences obtained for ITS PCR, identify of ≥ 99.6%, ≥ 94.3%, and ≥ 88.5% were considered, for species, genus, and family identification, respectively [20]. If there was a possibility of more than one species, identification was considered at the genus level. If there were species from different genera, the results were considered inconclusive.

Enzyme production by fungal isolates

Liquid state fermentation of selected isolates and production of hydrolytic enzymes

Liquid state fermentation was carried out for selected isolates according to McPhillips et al. [21] using the *Chaetomium thermophilum* minimal media (CTMM) recipe (5x) media containing KH_2PO_4 (15.0 g/L), K_2HPO_4 (10.0 g/L), $MgSO_4{\cdot}7H_2O$ (2.5 g/L), $CaCl_2{\cdot}2H_2O$ (0.5 g/L), yeast extract 0.1% ($^{w}/_{v}$) (5g), and corn steep liquor 150 mL with trace salts: (1000x) $FeSO_4{\cdot}7H_2O$ (0.005 g/L), $MnSO_4{\cdot}4H_2O$ (0.0016 g/L), $ZnSO_4{\cdot}7H_2O$ (0.0014 g/L), and $CoCl_2{\cdot}6H_2O$ (0.0002 g/L). The pH of the media was adjusted to 5.5 using concentrated acetic acid. The media was sterilized by autoclaving at 121 ℃ for 15 min. Fungal isolates were cultured in a 250 mL Erlenmeyer flask containing 80 mL of 2% (v/v) glucose and 20 mL of 5 × CTMM medium for biomass production. Pieces of the mycelial mat (4 × 1 cm^2) were removed from the outer regions of actively growing plates and transferred to flasks containing growth medium. After 2 days of incubation, fungal biomass was collected in sterile 50 mL tubes and used as a starter culture for the induction of enzymes. Induction media (100 mL) were prepared in 250 mL flasks containing 1 × CTMM, and a combination of sorghum, wheat bran, and oat bran (each at 0.75%) was used to induce production of cellulase, pectinase, xylanases, and mannanases. The pH of the media was adjusted to 5.5, and the media was sterilized by autoclaving at 121 ℃ for 15 min. Inducing experiments were performed by inoculating 10% (v/v) starter fungal culture to flasks and transferred to an incubator shaker set to rotate at 140 rpm at room temperature. Cultures were incubated in a medium for a period of 192 h, i.e., day 0 (6 h post-inoculation) until day 8. A fraction was taken from cultures on days 0, 1, 3, 4, 6, and 8 and stored at − 20 ℃ until further assays were conducted.

3, 5-Dinitrosalicylic acid (DNS) assay: determination of reducing sugar assay

The dinitrosalicylic acid (DNS) assay [22] was used to measure the reducing sugars released during enzyme assays with respect to positive controls, i.e., glucose, D-galacturonic acid, xylose, and mannose. The DNS solution was made up by combining two components—alkaline DNS reagent (solution A) and Rochelle salt (solution B). Solution A (alkaline DNS reagent): a 16 g quantity of NaOH was dissolved in 200 mL of distilled dH_2O, and the resultant solution was heated to 80–90 ℃. A 10 g amount of 3, 5-dinitrosalicyclic acid powder was gradually (very very slowly) added with constant stirring. Note: Having the NaOH solution hot induced rapid dissolution of DNS powder. Solution B (Rochelle salt): A solution of Rochelle salt was prepared by dissolving 300 g of potassium tartrate in 500 mL mH_2O; this solution was heated to between 50 and 60 ℃. Solution B was added to solution A in a 1-L volumetric flask (stored in a 60 ℃ oven until use) and cooled. The final volume was adjusted to 1 L with dH_2O. This DNS reagent was stable for 3–4 weeks while stored in a dark glass bottle.

Relative enzyme activity was measured by determining the amount of reducing sugars released following an incubation period of 15 min at 40 ℃ with 20 μL of crude extract and 230 μL of 1% (w/v) substrate. After the incubation period, 0.5 mL of 3, 5-DNS reagent was added to the sample tubes and boiled for 10 min. The absorbance at 540 nm was measured, and relative enzyme activities were expressed in international units (U), as the amount of enzyme that releases 1 µmol of glucose, galacturonic acid, xylose, or mannose per min per mL.

Reagents for enzyme assays were prepared as follows:

> Ammonium acetate (AmAc) buffer, 50 mM: the solution was made by dissolving 3.854 g of ammonium acetate in 1 L dH_2O and stored in a dark glass bottle. The solution pH was adjusted to 5.5 using concentrated acetic acid. Standards: glucose, D-galacturonic acid, xylose, and mannose were used as standards for DNS assay. Solutions of concentration 10 mg/mL were made up in AmAc buffer by dissolving 30 mg of powdered sugar in 30 mL of AmAc buffer. Substrate solutions (1% w/v): carboxymethylcellulose, citrus pectin, beechwood xylan, wheat arabinoxylan, locust bean gum, and guar gum. Each standard was made by dissolving 0.3 g of respective powders in 30 mL of AmAc buffer.

Bradford assay: determination of protein content (mg/ml)

Bradford assay was conducted for rapid determination of protein concentration in crude extracts collected over the course of 8 days. Aliquots of 50 μl of suitably diluted extract were combined with 50 μl of Bradford dye-binding reagent and incubated at room temperature for 5 min. Final absorbance values were read at 595 nm to estimate protein concentrations by reference to a standard curve of bovine serum albumin (BSA) (1–40 μg/ml) [23]. For each isolate, relative enzyme activity was normalized against the total protein content of crude extract (mg/ml) and presented as U/mg.

Antioxidant screening of fungal extracts

Preparation of crude fungal extracts for antioxidant assays

Fungal isolates were grown on fresh PDA plates at room temperature for 5 days. Three pieces (0.5 cm × 0.5 cm) of mycelial agar plugs were inoculated into 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth, incubated at room temperature for 3 weeks with rotation at 140 rpm in an incubator shaker [24]. After 3 weeks, the culture was filtered to separate the mycelial (cells) mass and liquid culture. Cell mass and liquid culture fractions were freeze-dried. One gram of freeze-dried sample was then transferred to a 30 mL centrifuge tube. To this tube, 10 mL of dichloromethane was added. The sample was vortexed thoroughly and transferred to a shaker for 1 h at room temperature. After 1 h, 10 mL of ethanol was added to the tube and mixed thoroughly followed by shaking at room temperature for 1 h. To achieve phase separation, 10 mL of water was added to this mixture followed by shaking at room temperature for 1 h. The mixture was then centrifuged at 13,000 rpm for 10 min to separate the two phases. The upper polar aqueous phase and lower non-polar dichloromethane phase were transferred to separate tubes and centrifuged until particulates were removed. The dichloromethane supernatant was then transferred to glass tubes, and the ethanol phase was transferred to a pre-weighed 30 mL tube. Dichloromethane extracts were evaporated under nitrogen, the ethanol phase was dried using a Biotage TurboVap® Classic LV Automated Evaporation System, and any residual water was freeze-dried. Approximately 0.2 g of non-polar fractions were reconstituted in 2 mL dichloromethane, and polar fractions were reconstituted in 2 mL ethanol and stored at −20 ℃ until used further for assays.

Oxygen radical absorbance capacity—ORAC assay

This assay is based on scavenging peroxyl radicals, generated by 2, 2'-azinobis (2-methylpropionamidine) dihydrochloride (AAPH), which cause degradation of fluorescein in the reaction mixture resulting in loss of fluorescence. If antioxidants are present in the test sample, they can interfere with the scavenging peroxyl radicals and consequently prevent loss of fluorescence [25]. The reaction was conducted in a black 96-well microtitre plate. Twenty microliters of standards/blank/

samples were pipetted in triplicates and pre-incubated with 120 μL of 0.117 μmol fluorescein in the plate reader at 37 ℃ for 15 min. Sixty microliters of 40 mM AAPH was added 15 min into the reaction, and fluorescence was read every minute for 2 h. The antioxidant curve (fluorescence versus time) is normalized to the corresponding curve of blanks by multiplying original data by the factor fluorescence $_{\text{blank, } t=0}$/fluorescence $_{\text{sample, } t=0}$. From the normalized curve, the area under the curve (AUC) is calculated: $[1+\sum_{i=1}^{i=n-1}\frac{f_i}{f_0}+\frac{f(x_n)}{f(x_0)}]$. The net AUC is calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC-FL values were expressed as µmol Trolox equivalents (TE) per gram of dried fungal extract based on the standard curve calculated for each assay.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

This assay uses the stable organic nitrogen radical DPPH* which is a deep purple color and is readily available. Reduction of DPPH* by the antioxidant species in the test sample results in loss of color is measured at 517 nm [26]. DPPH assay was conducted in 96-well micro-titer plates. A linear regression curve for standards was constructed and used to calculate the DPPH values for the samples in μmol Trolox equivalent per gram of dried fungal extract.

Ferric ion reducing antioxidant power (FRAP) assay

FRAP assay is based on the reduction of ferric ions to ferrous ions which results in the formation of a colored complex ferrous-tripyridyltriazine. A change in absorbance is measured at 593 nm in the test sample and compared to values obtained from standards containing ferric ions in known concentrations [27]. FRAP was conducted in 96-well micro-titer plates. A linear regression curve for Trolox standards was constructed and used to calculate the FRAP values of the samples as μmol Trolox equivalent (TE) per gram of dried fungal extract.

LC–MS analysis of fungal metabolites

Fungal extracts prepared for antioxidant assays were analyzed using LC–MS on an Agilent Technologies 1200 series pump system and an Agilent 6250 accurate mass Q-TOF instrument in the positive ion mode. A 35-min gradient, starting with 98% mobile phase A: water (0.1% v/v formic acid), to mobile phase B: 2% acetonitrile (0.1% v/v formic acid) was set to run through a ZORBAX SB-C18 (4.6×100 mm, 3.5 micron) column at a flow rate of 0.5 mL/min. A blank with 100% acetonitrile was run between each sample. The TOF–MS was equipped with a dual-nebulizer electrospray source, allowing the continuous introduction of reference mass compounds. The instrument was scanned from m/z 100 to 1700 for all samples. This mass range enabled the inclusion of two reference mass compounds, which produced ions at m/z 121.0508 and 922.0097. The injected sample volume was 5 μL. The remaining TOF conditions in positive ESI mode are shown in Table 2. A database of 514 metabolites and mycotoxins was created in Excel from reference sources [28, 29]. The compounds were added to the Agilent formula database generator to create a usable file against which the ions generated from the analysis were screened. The metabolite list consisted of compounds belonging to classes—Trichothecenes, Aflatoxins and their precursors, Ochratoxins, Sphiganin mycotoxins, Cytochalasins, Ergot amines and related alkaloids, Atranones, Peptides, and other metabolites. All data analysis was performed using the Agilent Mass Hunter Qualitative workflow software. Total ion chromatograms and extracted ion chromatograms of metabolites were recorded and collated for each extract. An Excel spreadsheet with the mass of 514 fungal metabolites and mycotoxins was constructed where the monoisotopic mass of each compound was calculated using their molecular formula. This file was converted to CSV (comma-separated values) format for use by the Agilent Mass Hunter data analysis software. The data analysis editor of the software allows for a selection of adducts such as H^+, NH_4^+, and Na^+ and neutral loses to be searched automatically when analyzing the compound in positive electrospray mode. For this study, the main criteria of detection were based on mass accuracy. The LC–MS was calibrated with a reference mix with compounds of known accurate mass (lock-mass) and was used as a way to calibrate the m/z value of the observed mass of compounds [30]. The mass tolerance was set to 1 ppm, and the minimum peak intensity was set to 1000 counts. These two criteria were successfully used for the identification of compounds with accurate mass.

Results

Isolation and identification of fungal isolates

Fungi collected from Moylish Park, Curraghchase Forest (Co. Limerick, Ireland), and woodlands in Scariff (Co. Clare, Ireland) were brought back into the laboratory and isolated on PDA plates. Isolates were cultured at 28 ℃ for 3–5 days until the formation of fungal colonies was visualized. Mycelial colonies were removed from the plate using sterile tips and transferred onto new PDA plates to purify individual fungal strains. To identify the respective strains in the biobank, DNA was extracted from purified isolates. PCR was conducted on extracted DNA using rDNA primers ITS1 and ITS4 [18]. Following the purification of PCR products, DNA sequencing was performed to identify the fungi.

The raw DNA sequence information was analyzed using the NCBI-BLAST search engine. The BLAST search method matches sequences in the online GenBank database to the query sequence entered by the user and generates a list of closely matched sequences from the GenBank database [19]. Details of the percentage identity of the isolated fungi to GenBank sequences are given in Table 1.

Of 83 fungi isolated and identified by DNA sequencing, the most common were species belonging to genera *Trichoderma* and *Mucor* with 27 and 23 species present, respectively. Other genera present included six *Fusarium* species; four species each were identified as *Hypocrea, Hypomyces*, and *Penicillium*; three fungi were identified as *Clonostachys* species while two were identified as *Hypholoma fasciculare*. The remaining fungi were identified as *Bjerkandera adusta*, *Diatrypella* sp. strain *MNU97*, *Didymella fabae* strain JH3J4-5, *Epicoccum nigrum*, *Lecanicillium* sp. strain UFSMQ06, *Rhizoctonia* sp. 5170, *Trametes gibbosa* strain ZBS2012 & *Talaromyces minioluteus* culture CBS:996.72. *Trichoderma, Fusarium, Hypocrea, Hypomyces, Penicillium, Clonostachys, Diatrypella, Didymella, Epicoccum, Lecanicillium*, and *Talaromyces* species belong to Ascomycota phylum while *Hypholoma, Bjerkandera, Rhizoctonia*, and *Trametes* species belong to the phylum Basidiomycota. *Mucor* species belong to the phylum Mucoromycota. These sequences have been submitted to GenBank accession numbers OR350933 to OR351016. Of the 83 fungi, 23 were chosen for further studies as they had not previously been studied in the literature for bioactivities (Table S1 in Supplementary Materials). A phylogenetic tree representing 23 fungal isolates is presented in Fig. 1.

These strains were chosen for their potential to produce useful bioactive compounds and enzymes of industrial importance as outlined here. There is some literature citing data on potential bioactivities for *Mucor* species. The antioxidant potential of *Mucor circinelloides* has been recorded by Hameed et al. showing the reported *Mucor* strains to be rich sources of antioxidants and secondary metabolites [31]. A study performed on 30 *Trichoderma* species isolated from a marine environment in South Korea demonstrated several strains to have high radical scavenging and notable tyrosinase activity [32]. They have also been evaluated as potential biocontrol agents as their biological processes lead to the production of cell wall degrading enzymes such as chitinases, glucanases, mannanases, and proteases aiding them in mycoparasitism [33–35]. *Clonostachys rosea* has been previously reported as a biocontrol agent inhibiting the growth of plant pathogenic fungi [36, 37]. Isolated species for *Fusarium* have been reported production of enzymes such as xylanase, amylase, and β-glucosidase highlighting their usefulness for industrial and environmental applications [38–40]. Regarding *Hypomyces* species, there is only one study that was performed to assess the bioactivity of *Hypomyces chrysospermus*. It reported excellent antimicrobial activity, moderate antioxidant, and anticancer activity [41]. Fungi of the genus *Epicoccum* are mainly known for their use as biocontrol agents; however, there is great interest in the metabolites of these fungi [42, 43]. Secondary metabolites of an isolated *E. nigrum* P16 strain have been shown to produce compounds with antifungal activity [44].

Presented here are the results of enzyme, antioxidant activities, and secondary metabolite profiles from 9 of the 23 fungal isolates (Table 2).

Determination of hydrolytic enzyme activities of fungal isolates

The hydrolytic enzyme activity for each isolate was recorded against six substrates: carboxymethyl cellulose, citrus pectin, beechwood xylan, wheat arabinoxylan, locust bean gum, and guar gum. The synergistic action of several hydrolytic enzymes is required to breakdown such complex substrates. The relative enzyme activities have been displayed as (U/mg) normalized against the protein content of crude extract for fractions collected over the course of 8 days. The cellulase activities (U/mg) of all tested fungal isolates can be seen in Fig. S1. The highest amount of cellulase activity was found in culture media from *Penicillium expansum* (F2) with 25.2 U/mg on day 1, while *Trichoderma koningii* (F9) with 18.3 U/mg on day 6 was the next best cellulase producer.

Pectinase activity for all isolated fungi against citrus pectin is presented in Fig. S2. *Penicillium expansum* (F2) recorded the highest activity on day 4 at 112.5 U/mg. *Trichoderma koningii* (F9) showed moderate pectinase activity of 54 U/mg at day 2. *Mucor luteus* (F6) showed moderate activity in the range of 30–45 U/mg in all fractions collect-ed after the first 24-h incubation period, with the maximum activity of 44 U/mg on day 4. Of all the six fractions from *Clonostachys rosea* (F1), only the fraction collected on day 6 had moderate pectinase activity of 34 U/mg.

The results of potential extracellular β-xylanase enzyme activity are shown in Fig. S3. *Clonostachys rosea* (F1) and *Trichoderma koningii* (F9) were the highest β-xylanase producers with activity of 351.63 U/mg on day 3 and 372.15 U/mg on day 6, respectively. *Fusarium tricinctum* (F4) and *Fusarium avenaceum* (F3) showed moderate enzyme activity of 146.78 U/mg on day 3 and 139.36 U/mg on day 4, respectively.

In Fig. S4, the arabinoxylanase activity of all isolates against 1% (w/v) wheat arabinoxylan is shown. *Clonostachys rosea* (F1) and *Trichoderma koningii* (F9) reported

Table 1 Biobank of fungal isolates

Top BLAST hit	GenBank ID of top hit	% Identity of sequence to top BLAST hit	Location where fungus was isolated from	Fungal ID in Biobank	GenBank Accession number
Trichoderma sp. isolate 12–65	MT035967.1	99.45	Curraghchase Forest	95	OR351009
Trichoderma sp.	MT035967.1	98.46	Moylish Park	1	OR350933
Lecanicillium sp. strain UFSMQ06	KX496884.1	98.64	Woodland, Scariff	88	OR351002
Trichoderma atroviride isolate T-4–5	MT341775.1	100.00	Moylish Park	4	OR350936
Trichoderma sp. isolate SDAS204074	MK870578.1	100.00	Moylish Park	7	OR350939
Trichoderma sp. isolate PSW2-1	KU727803.1	100.00	Woodland, Scariff	22	OR350948
Trichoderma shangrilaense strain WT34012	MH287490.1	100.00	Woodland, Scariff	23	OR350949
Trichoderma sp. isolate SDAS204074	MK870578.1	100.00	Woodland, Scariff	33	OR350958
Trichoderma sp. isolate SDAS204074	MK870578.1	100.00	Woodland, Scariff	35	OR350960
Trichoderma polysporum culture CBS:261.58	MH857774.1	100.00	Woodland, Scariff	37C	OR350961
Trichoderma asperellum isolate y-081	MN227551.1	100.00	Woodland, Scariff	49	OR350973
Trichoderma viridescens isolate C01-L2	MK804405.1	100.00	Woodland, Scariff	52	OR350975
Trichoderma asperellum isolate 77J14	KU516601.1	100.00	Woodland, Scariff	54	OR350977
Trichoderma asperellum isolate y-081	MN227551.1	100.00	Woodland, Scariff	58	OR350980
Trichoderma sp. 3 RJ-2019 strain 457E	MZ078769.1	100.00	Woodland, Scariff	61B	OR350982
Trichoderma asperellum isolate y-081	MN227551.1	100.00	Woodland, Scariff	62C	OR350983
Trichoderma sp. 3 RJ-2019 strain 457E	MZ078769.1	100.00	Woodland, Scariff	63	OR350984
Trichoderma viride isolate C03-L3	MK804406.1	100.00	Woodland, Scariff	73	OR350990
Trichoderma atroviride strain LSK_7	MT514373.1	100.00	Woodland, Scariff	80	OR350995
Trichoderma sp. isolate SDAS203372	MK870853.1	99.81	Woodland, Scariff	85	OR350999
Trichoderma koningii strain QLF37	FJ025200.1	100.00	Woodland, Scariff	90	OR351004
Trichoderma sp. isolate SDAS203372	MK870853.1	100.00	Curraghchase Forest	93	OR351007
Trichoderma sp. isolate SDAS203372	MK870853.1	99.82	Curraghchase Forest	99	OR351011
Trichoderma sp. SM13-21–20-4	MN905867.1	100.00	Curraghchase Forest	101	OR351012
Trichoderma sp. isolate Gen 9	KY040278.1	100.00	Curraghchase Forest	103	OR351013
Trichoderma sp. INBio3692E	KX858890.1	99.32	Woodland, Scariff	11	OR350943
Trichoderma viride	HM776435.1	100.00	Rotting coconut	105	OR351015
Mucor hiemalis f. hiemalis IFM 47742	LC413619.1	100.00	Moylish Park	2	OR350934
Mucor hiemalis f. hiemalis culture CBS:978.68A	MH859259.1	99.65	Woodland, Scariff	17	OR350945

Table 1 (continued)

Top BLAST hit	GenBank ID of top hit	% Identity of sequence to top BLAST hit	Location where fungus was isolated from	Fungal ID in Biobank	GenBank Accession number
Mucor sp. NFL5	JX160050.1	98.60	Woodland, Scariff	21	OR350947
Mucor abundans isolate REB-017A	MK164150.1	100.00	Woodland, Scariff	28	OR350953
Mucor hiemalis isolate ARMI-26	MH794214.1	100.00	Woodland, Scariff	34	OR350959
Mucor hiemalis f. silvaticus isolate P1288	OK584538.1	100.00	Woodland, Scariff	38y	OR350962
Mucor hiemalis f. silvaticus strain CBS 412.71	JN206124.1	100.00	Woodland, Scariff	40	OR350964
Mucor sp. isolate ARMI-10	MH794198.1	100.00	Woodland, Scariff	42	OR350966
Mucor hiemalis f. silvaticus strain CBS 412.71	JN206124.1	100.00	Woodland, Scariff	43	OR350967
Mucor hiemalis isolate ARMI-26	MH794214.1	99.83	Woodland, Scariff	44	OR350968
Mucor hiemalis strain FIF_4	MT514377.1	99.83	Woodland, Scariff	45	OR350969
Mucor luteus culture CBS:567.70A	MH859851.1	99.82	Woodland, Scariff	46	OR350970
Mucor sp. isolate ARMI-10	MH794198.1	100.00	Woodland, Scariff	47	OR350971
Mucor leutus strain FSU 6171 clone 1	EU484251.1	100.00	Woodland, Scariff	48	OR350972
Mucor hiemalis isolate 146	JX535127.1	100.00	Woodland, Scariff	51	OR350974
Mucor abundans isolate REB-017A	MK164150.1	100.00	Woodland, Scariff	53	OR350976
Mucor hiemalis isolate RP242_4A	KX067818.1	99.66	Woodland, Scariff	65y	OR350986
Mucor hiemalis strain QH09-03	MT072072.1	99.83	Woodland, Scariff	75	OR350991
Mucor hiemalis strain QH09-03	MT072072.1	100.00	Woodland, Scariff	76	OR350992
Mucor abundans strain P532	MZ078786.1	100.00	Woodland, Scariff	78	OR350994
Mucor hiemalis strain QH09-03	MT072072.1	100.00	Woodland, Scariff	81	OR350996
Mucor hiemalis strain LSK_3	MT514370.1	99.77	Woodland, Scariff	86	OR351000
Mucor hiemalis isolate YA-11	MT626048.1	100.00	Curraghchase Forest	92	OR351006
Fusarium sp. HMA-12	GU480951.1	98.14	Moylish Park	6	OR350938
Fusarium avenaceum isolate HHC-FA73	OP117234.1	100.00	Moylish Park	9	OR350941
Fusarium tricinctum strain ReFu	OQ975669.1	100.00	Moylish Park	10	OR350942
Fusarium tricinctum strain ReFu	OQ975669.1	100.00	Woodland, Scariff	15	OR350944
Fusarium cerealis strain Carm4	MG979797.1	100.00	Woodland, Scariff	60	OR350981
Fusarium cerealis strain Carm4	MG979797.1	100.00	Woodland, Scariff	64	OR350985
Hypocrea sp. SM13-18–6-1	MN905771.1	97.40	Woodland, Scariff	77	OR350993
Hypocrea pachybasioides strain CBS 119319	FJ860796.1	100.00	Curraghchase Forest	94	OR351008
Hypocrea pachybasioides strain Ds-6Pc-1–6-2–2	KJ191334.1	100.00	Curraghchase Forest	96	OR351010

Table 1 (continued)

Top BLAST hit	GenBank ID of top hit	% Identity of sequence to top BLAST hit	Location where fungus was isolated from	Fungal ID in Biobank	GenBank Accession number
Hypocrea pachybasioides strain CBS 119319	FJ860796.1	100.00	Curraghchase Forest	104	OR351014
Hypomyces armeniacus isolate PBM4214	MT237489.1	100.00	Woodland, Scariff	24	OR350950
Hypomyces armeniacus	FN859424.1	100.00	Woodland, Scariff	25	OR350951
Hypomyces armeniacus genomic DNA, strain TFC 02–86/2	FN859424.1	100.00	Woodland, Scariff	32	OR350957
Hypomyces armeniacus	OP179782.1	99.62	Woodland, Scariff	55	OR350978
Penicillium expansum isolate PF-022	MN752156.1	100.00	Moylish Park	5	OR350937
Penicillium crustosum voucher Pen c10	MT298917.1	100.00	Woodland, Scariff	29B	OR350954
Penicillium fimorum isolate 2011F12	MT558942.1	100.00	Woodland, Scariff	30	OR350955
Penicillium sp. *i*solate NP24-3–5	MW764444.1	100.00	Woodland, Scariff	39	OR350963
Clonostachys rosea culture CBS:376.55	MH869057.1	100.00	Moylish Park	3	OR350935
Clonostachys rosea culture CBS:128,889	MH865141.1	100.00	Woodland, Scariff	20	OR350946
Clonostachys sp. 1 RJ2014 strain 33914aDRJ	MF782758.1	100.00	Woodland, Scariff	89	OR351003
Hypholoma fasciculare isolate GPS 106	MK050597.1	99.84	Woodland, Scariff	31	OR350956
Hypholoma fasciculare isolate GPS 106	MK050597.1	100.00	Woodland, Scariff	70	OR350989
Mariannaea camptospora culture CBS:216.73B	MH860669.1	100.00	Moylish Park	8	OR350940
Mariannaea camptospora culture CBS:216.73B	MH860669.1	100.00	Curraghchase Forest	91	OR351005
Bjerkandera adusta	MF120203.1	100.00	Woodland, Scariff	69	OR350988
Diatrypella sp. strain MNU97	KX828167.1	100.00	Woodland, Scariff	84	OR350998
Didymella fabae strain JH3J4-5	KP117284.1	100.00	Woodland, Scariff	67	OR350987
Epicoccum nigrum	MT573480.1	100.00	Woodland, Scariff	87B	OR351001
Rhizoctonia sp. 5170	HQ853687.1	100.00	Silage, Cork	106	OR351016
Talaromyces minioluteus culture CBS:996.72	MH860641.1	99.81	Woodland, Scariff	57	OR350979

Samples collected at Moylish Park, Limerick V94 EC5T County Limerick, Scariff, County Clare, and Curraghchase Forest Park, County Limerick, Ireland

the highest enzyme activity at 645.50 U/mg on day 3 and 655.88 U/mg on day 6, respectively. *Fusarium avenaceum* (F3) showed competitive enzyme activity with 321 U/mg on day 3. In comparison, *Penicillium expansum* (F2) expressed slightly less than moderate activity of 153 U/mg on day 8.

The mannan components of the plant polymers are effectively broken-down using enzymes known as mannanases. Figure S5 shows the galacto-mannanase activity recorded for all isolates over an 8-day period. *Fusarium avenaceum* (F3) was the highest producer of galacto-mannanase against locust bean gum with 52 U/mg recorded on day 6. In comparison, *Clonostachys rosea* (F1) had moderate enzyme activity of 25 U/mg on day 3.

Mannanase activity was screened using 1% (w/v) guar gum as substrate. The enzyme activity of all isolates can be seen in Fig. S6. *Fusarium avenaceum* (F3) reported the highest mannanase activity at 45 U/mg on day 6 among all isolates. *Penicillium expansum* (F2) showed

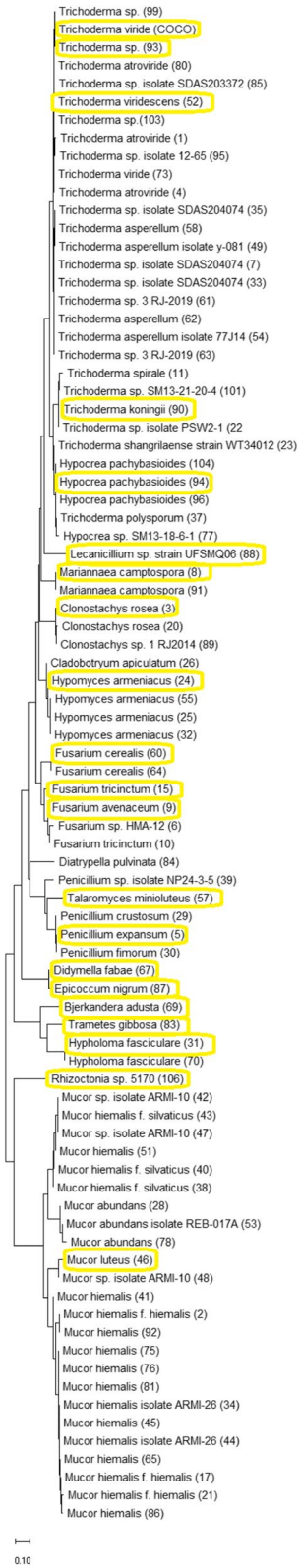

Fig. 1 Phylogenetic tree based on the internal transcribed spacer rDNA sequences isolated from fungal strains. Yellow highlighted fungi illustrate those selected for bioactivity screening

Table 2 Fungal strains screened for enzyme, antioxidant activities, and secondary metabolites

ID	Isolate name	Collection site
F1	*Clonostachys rosea*	Moylish Park
F2	*Penicillium expansum*	Moylish Park
F3	*Fusarium avenaceum*	Woodland, Scariff
F4	*Fusarium tricinctum*	Woodland, Scariff
F5	*Hypomyces armeniacus*	Woodland, Scariff
F6	*Mucor leutus*	Woodland, Scariff
F7	*Fusarium cerealis*	Woodland, Scariff
F8	*Epicoccum nigrum*	Woodland, Scariff
F9	*Trichoderma koningii*	Woodland, Scariff

mannanase enzyme activity around 35 U/mg on day 1. Moderate activity was seen in *Trichoderma koningii* (F9) with 18 U/mg on day 4.

Determination of antioxidant ability of crude fungal extracts

The oxygen radical absorbance capacity (ORAC) assay uses a biologically relevant radical source and therefore is one of the most relevant assays used to screen for natural anti-oxidants [45]. The reactions take longer in contrast to 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing ability of plasma (FRAP) assays; however, it gives a better indication of the action of antioxidants in a biological setting. The DPPH assay works on the mechanism of electron transfer where the extract is tested for its ability to scavenge the methanolic DPPH radical. Finally, FRAP works using a redox mechanism where if a sample has a lower redox potential than the reduction potential of FeIII/FeII, then it acts as an anti-oxidant [46]. Assessment of antioxidant activity is usually carried out by establishing the degree of antioxidant power calculated based on colorimetric and fluorometric reactions. Therefore, the assay's ability to generate an accurate antioxidant profile depends on several different factors namely species used, nutrient source of culture medium, and cultivation parameters [47].

There is limited literature available on the ORAC, DPPH, and FRAP activity of fungal strains presented in this study. In the ORAC assay, if antioxidants are present in the tested extract, they will prevent the degradation of fluorescein in the reaction mixture and consequently prevent loss of fluorescence which is correlated to the antioxidant units as micromole Trolox equivalents (TE) per gram of sample (dried fungal extract reported here) based on the standard curve calculated for each assay [25]. Figure 2 shows the ORAC activity values for the polar and non-polar extracts of liquid culture and cell

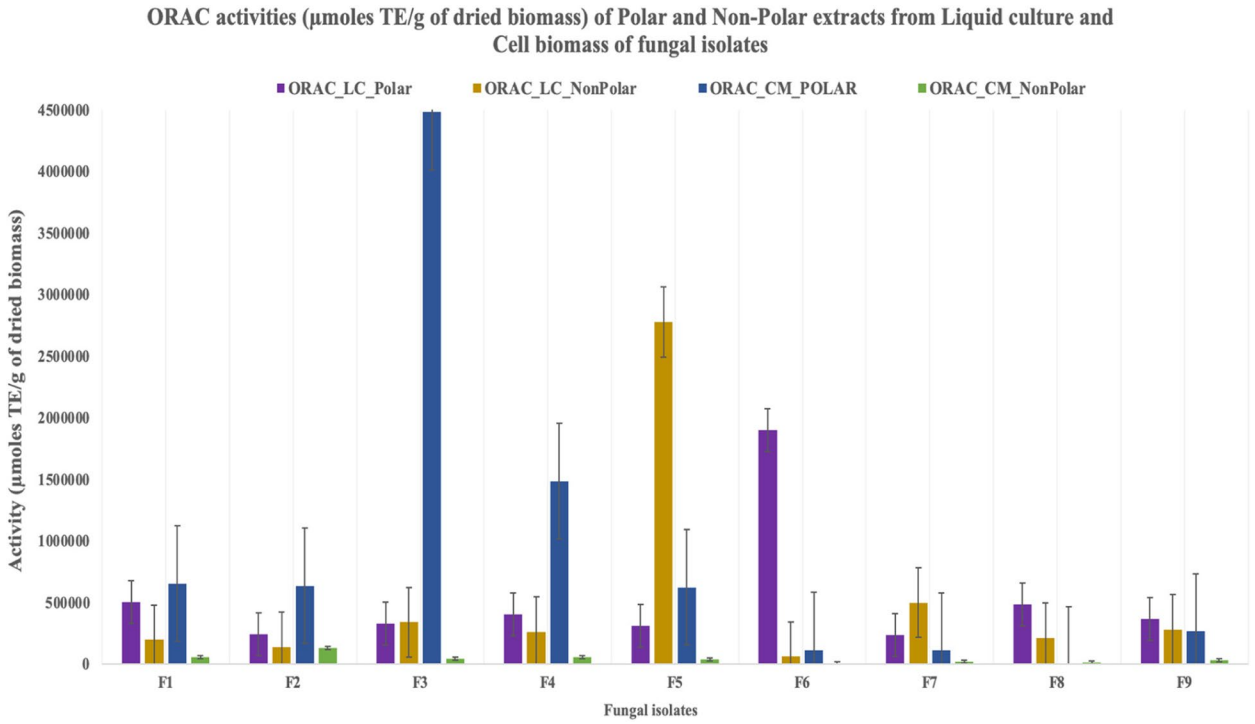

Fig. 2 ORAC activities (µmol TE/g of dried fungal biomass) of polar and non-polar extracts from cell biomass (CM) and liquid culture (LC) of fungal isolates F1: *Clonostachys rosea*, F2: *Penicillium expansum*, F3: *Fusarium avenaceum*, F4: *Fusarium tricinctum*, F5: *Hypomyces armeniacus*, F6: *Mucor luteus*, F7: *Fusarium cerealis*, F8: *Epicoccum nigrum*, and F9: *Trichoderma koningii*. Error bars represent the SD of test triplicates

biomass. The highest ORAC activity was recorded for the polar cell biomass extract of *Fusarium avenaceum* (F3) isolate at 4,488,035 µmol TE/g of dried biomass. The second highest ORAC activity was a non-polar extract from a liquid culture of isolate *Hypomyces armeniacus* (F5) at 2,779,609 µmol TE/g. The polar cell biomass extracts of *Clonostachys rosea* (F1), *Penicillium expansum* (F2), and *Hypomyces armeniacus* (F5) isolates with 655,669 µmol TE/g, 636,889 µmol TE/g, and 626,670.65 µmol TE/g ORAC activity, respectively, also demonstrated good antioxidant levels. For five fungal isolates, the polar extracts from cell biomass showed higher activity compared to non-polar extracts.

In the DPPH assay, the loss of color of stable organic nitrogen radical DPPH* by the antioxidant species present in the test sample was measured and DPPH values were expressed as µmol Trolox equivalent per gram of dried fungal extract. Figure 3 displays the DPPH activity of polar and non-polar extracts of liquid culture and cell biomass of all fungal isolates calculated to 1 g of dried biomass of fungi as per assay conditions. The highest activity was recorded for the non-polar liquid culture extracts of *Trichoderma koningii* (F9) with 30,676 µmol TE/g followed by 22,646 µmol TE/g for *Fusarium avenaceum* (F3) and 22,558 µmol TE/g for polar liquid culture extract of *Trichoderma koningii* (F9). Both polar and non-polar liquid culture extract of *Fusarium cerealis* (F7) showed comparable activity at 15,558 µmol TE/g and 15,712 µmol TE/g, respectively. Moderate DPPH activity was recorded for polar liquid culture extracts of *Fusarium tricintum* (F4), *Clonostachys rosea* (F1), and non-polar liquid culture extracts of *Epicoccum nigrum* (F8) with 13,725 µmol TE/g, 12,572 µmol TE/g, and 13,700 µmol TE/g, respectively. Both polar and non-polar liquid culture extracts of *Penicillium expansum* (F2) showed similar DPPH activities at 10,141 µmol TE/g and 10,658 µmol TE/g, respectively. Only two polar cell biomass extracts showed DPPH activity of which isolate *Epicoccum nigrum* (F8) showed a moderate activity of 15,469 µmol TE/g while no DPPH activity was recorded for the corresponding non-polar cell biomass extracts for any isolate.

The FRAP assay measures radical scavenging activity through the reduction of ferric ions to ferrous ions which results in the formation of a colored complex ferrous-tripyridyltriazine. The activity was measured for both polar and non-polar extracts of cell biomass and liquid culture medium of all fungal isolates and expressed as µmol Trolox equivalent per gram of dried fungal extract (Fig. 4). Polar liquid culture extract of *Mucor luteus* (F6)

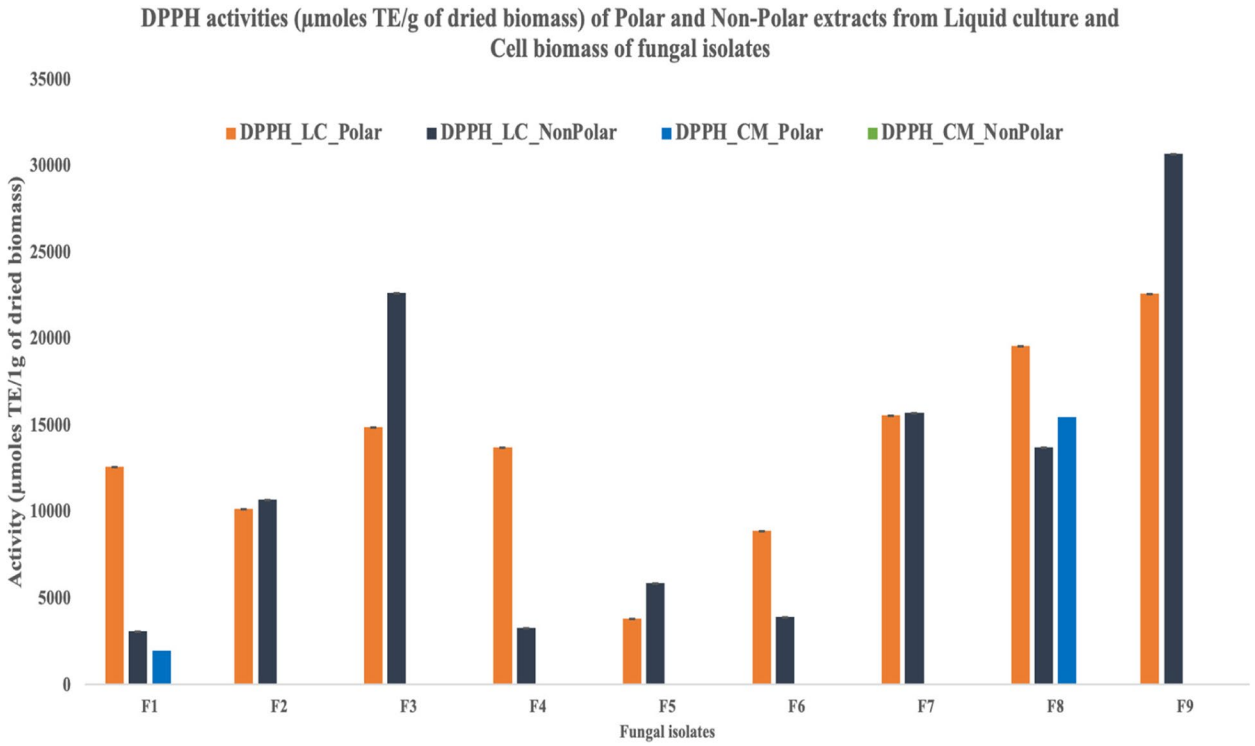

Fig. 3 DPPH activities (µmol TE/g of dried fungal biomass) of polar and non-polar extracts from cell biomass (CM) and liquid culture (LC) of fungal isolates F1: *Clonostachys rosea*, F2: *Penicillium expansum*, F3: *Fusarium avenaceum*, F4: *Fusarium tricinctum*, F5: *Hypomyces armeniacus*, F6: *Mucor luteus*, F7: *Fusarium cerealis*, F8: *Epicoccum nigrum*, and F9: *Trichoderma koningii*. Error bars represent the SD of test triplicates

and *Epicoccum nirgrum* (F8) and cell biomass extract of *Fusarium avenaceum* (F3) showed highest FRAP activities at 82,144 µmol TE/g, 79,224 µmol TE/g, and 69,325 µmol TE/g, respectively. Liquid culture polar extracts of *Clonostachys rosea* (F1), *Fusarium tricintum* (F4), *Trichoderma koningii* (F9), and *Fusarium cerealis* (F7) recorded moderate FRAP activity at 36,019 µmol TE/g, 23,495 µmol TE/g, 25,812 µmol TE/g, and 23,352 µmol TE/g, respectively.

Analysis of secondary metabolite profile using LC–MS

Based on the fungal metabolite screening method for a database of 474 mycotoxins and fungal metabolites using LCMS (25) and an LC–MS/MS method for detecting cyclic dipeptides in tea [29], an LC–MS/MS protocol was established which is a simple and rapid method to analyze and identify key metabolites and mycotoxins present in fungal cultures. The extracellular polar extract of each isolate previously employed in screening for antioxidant activity was investigated for secondary metabolites and is listed in Table S11 in Supplementary Materials. The structure of the top three metabolites presented for all nine isolates are shown in Fig. 5. Based on the ORAC, DPPH, and FRAP results, it was concluded that polar fractions, specifically from the liquid culture exhibited some level of antioxidant activity for all isolates and were suitable for use in the LC–MS method development and analysis. Fungi often produce more than 100 secondary metabolites in rich growth media and to achieve complete resolution of all individual metabolites and media components is very difficult [48]. The best choice of separation for metabolites is often reverse-phase chromatography since its polarity is suitable for most secondary metabolites [48]. The use of the reverse phase C18 column for the separation of crude ethanolic extracts of isolates resulted in the detection of compounds such as small acids, alcohols, cyclic peptides, alkaloids, and anthraquinones as well as mycotoxins.

The most abundant metabolites detected among fungal extracts were benzoic acid; Cyclopiazonic acid; Jasmonic acid and its derivatives; mycotoxins Tenuazonic acid, Cyclopaldic acid, Fusaric acid, and prenitremone A; and cyclic peptides. Benzoic acid was present in *Clonostachys rosea* (F1), *Penicillium expansum* (F2), and *Fusarium tricinctum* (F4) and has not previously been reported for these strains. Benzoic acid is an aryl metabolite synthesized from L-phenylalanine and acts as a precursor to a wide variety of natural products such as hydroquinone, catechol, and vanillin [49]. Moreover, a study performed

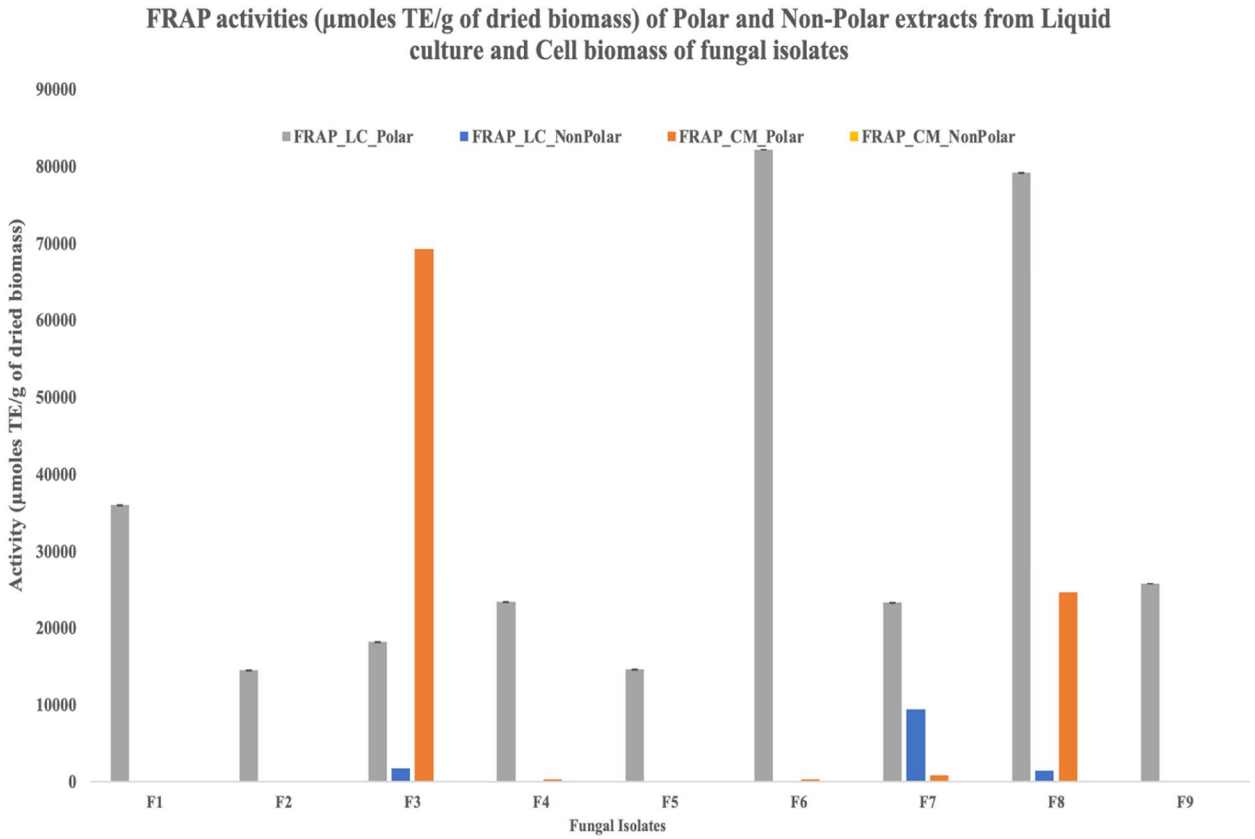

Fig. 4 FRAP activities (µmol TE/g of dried fungal biomass) of polar and non-polar extracts from cell biomass (CM) and liquid culture (LC) of fungal isolates F1: *Clonostachys rosea*, F2: *Penicillium expansum*, F3: *Fusarium avenaceum*, F4: *Fusarium tricinctum*, F5: *Hypomyces armeniacus*, F6: *Mucor luteus*, F7: *Fusarium cerealis*, F8: *Epicoccum nigrum*, and F9: *Trichoderma koningii*. Error bars represent the SD of test triplicates

on polish mushrooms for antibacterial, antiradical potential reported the presence of benzoic acid in the extracts of mushrooms with high phenolic content [50]. Cyclopiazonic acid, which is an indole tetramic acid, known to have antioxidant activity [51], was detected in *Hypomyces armeniacus* (F5). Other novel metabolites identified in extracts of isolates in this study include compounds Jasmonic acid and its derivatives which were found for isolates *Fusarium cerealis* (F7) and *Epicoccum nigrum* (F8). These plant growth hormones have previously been reported in several fungi [52, 53]. *p*-anisaldehyde was reported in *Fusarium avenaceum* (F3). In the white-rot basidiomycete *Pleurotus ostreatus* the biosynthesis of *p*-anisaldehyde has been reported as playing a role in generating H_2O_2-activated peroxidase in the lignin-degradation system. The extract containing *p*-anisaldehyde from *Pleurotus ostreatus* exhibited antimicrobial activity against *Bacillus subtilis, Pseudomonas aeruginosa, Aspergillus niger,* and *Fusarium oxysporum* [54]. However, this compound has not been reported previously in *Fusarium avenaceum*.

Mycotoxins were also detected in fungal extracts: Tenuazonic acid in *Fusarium cerealis* (F7); Cyclopaldic acid, in *Mucor luteus* (F6), *Fusarium tricinctum* (F4), and *Trichoderma koningii* (F9); and Fusaric acid in *Hypomyces armeniacus* (F5) and prenitremone A in *Penicillium expansum* (F2). Tenuazonic acid is a mycotoxin isolated from species of *Aspergillus*, *Penicillium*, and *Fusarium* [55] and an extract from *Alternaria alternate* containing tenuazonic acid has demonstrated promising antitubercular activity against *Mycobacterium tuberculosis* [56]. Cyclopolic and cyclopaldic acids have been reported previously in *Penicillium cyclopium* [57], but this is the first report to the authors' knowledge of this compound in *Mucor luteus* (F6), *Fusarium tricinctum* (F4), and *Trichoderma koningii* (F9). The mycotoxin Citreoviridin and the antibiotic Canescin were both detected in *Trichoderma koningii* (F9) each, both of which have not previously been reported for this fungus. *Penicillium citreonigrum* has been reported to produce the mycotoxin Citreoviridin [58], while the antibiotic Canescin is produced by *Penicillium canescens* [59].

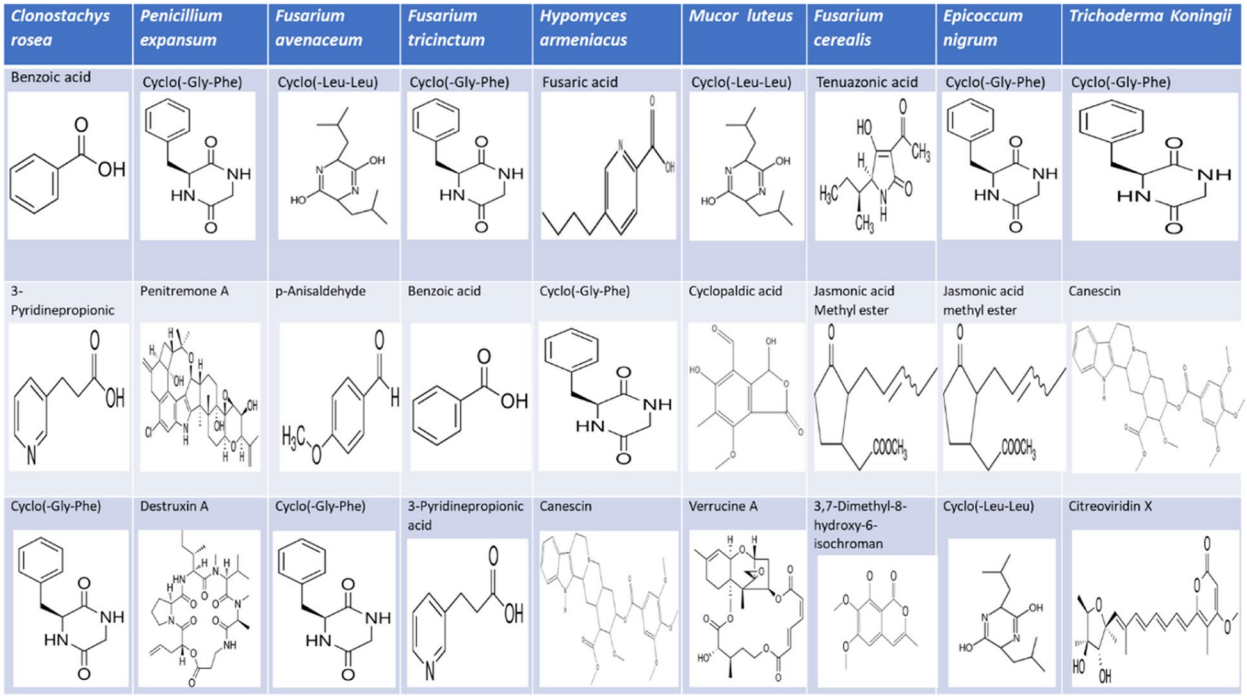

Fig. 5 Top three secondary metabolites of extracts from fungi cultivated with potato dextrose broth for 21 days. Fungal isolates F1: *Clonostachys rosea*; F2: *Penicillium expansum*; F3: *Fusarium avenaceum*; F4: *Fusarium tricinctum*; F5: *Hypomyces armeniacus*; F6: *Mucor leutus*; F7: *Fusarium cerealis*; F8: *Epicoccum nigrum*; and F9: *Trichoderma koningii*

Metabolites with high abundance among fungal strains were the cyclic dipeptides. Cyclic dipeptides are synthesized from amino acids of organisms as secondary metabolites of side products of terminal peptide cleavage. They are biosynthesized via either tRNA-dependent cyclodipeptide synthases or non-ribosomal peptide synthases [60]. As a large class of secondary metabolites, they are abundant in bacteria, fungi, plants, and animals [61]. One of the most common dipeptides encountered in this study was Cyclo(-Gly-Phe) found in isolates *Clonostachys rosea* (F1), *Penicillium expansum* (F2), *Fusarium avenaceum* (F3), *Fusarium tricinctum* (F4), *Hypomyces armeniacus* (F5), *Epicoccum nigrum* (F8), and *Trichoderma koningii* (F19). Another example of an identified dipeptide was Cyclo(-Leu-Leu) found in isolates *Fusarium avenaceum* (F3), *Fusarium tricinctum* (F4), *Mucor leutus* (F6), and *Fusarium cerealis* (F7). While there is no specific literature citing the presence of these cyclic dipeptides in isolates, these compounds are distributed among bacteria, fungi, plants, and animals. Refer to Tables S2–S10 in Supplementary Material for individual metabolite profiles.

Discussion

All enzymes screened in this study have potential uses in industries such as pulp paper, bread, and baking, and treatment of agricultural waste to produce value-added products [4, 62, 63]. Isolate F2: *P. expansum* reported 112.5 U/mg pectinase activity against citrus pectin and 25 U/mg cellulase activity against carboxymethylcellulose. While the activities reported in this study are from crude extracts, further optimization of growth conditions and purification can enhance these strains' abilities to produce enzymes rivaling those reported in the literature.

Isolate *Trichoderma koningii* (F9) showed high β-xylanase and arabinoxylanase enzyme activity. To the best of the authors' knowledge, there is no literature on the quantitative arabinoxylanase activity for this *Trichoderma* species. This is the first study to measure high arabinoxylanase activity at 655.8 U/mg for isolate *Trichoderma koningii* (F9) as the first account of this enzyme activity by strains isolated from Irish habitats. This current study also recorded β-xylanase production at 372.15 U/mg for isolate *Trichoderma koningii* (F9). β-xylanase and arabinoxylanase enzyme activity by *Clonostachys rosea* (F1) at 351.63 U/mg and 645.50 U/mg and arabinoxylanase activity for isolate *Fusarium avenaceum* (F3) at 139.36 U/mg were some of the highest among all isolates. The genome of *Clonostachys rosea* has been studied extensively, and while it contains many putative genes encoding plant biomass degradation enzymes, little is reported in terms of their functional characterization [64]. While there is little to no literature on the arabinoxylanase activity of *Fusarium* species, some isolates of this

genus have previously reported the production of xylanase enzymes [38, 65–67]. Enzymes such as xylanases are especially high in industrial demand, e.g., in bio-pulping treatment of wood chips and other lignocellulose materials prior to mechanical or chemical pulping of wood to produce paper. Using fungal xylanases is a feasible and cost-effective method that increases the strength properties of paper and reduces environmental expenses [68]. Xylanases applied in the pulp and paper industry will be worth over USD 35 million by 2024 [69].

Of all the isolates in this study, *Fusarium avenaceum* (F3) reported the highest mannanase and galactomannanase activity of 45.03 U/mg and 52.33 U/mg, respectively, for the crude fungal extracts. Endo β-1, 4 mannanases have been successfully obtained from filamentous fungi such as *Aspergillus niger* and *Aspergillus flavus*. Kote et al. reported 40.011 IU/ml of mannanase activity for *A. niger* and 33.532 IU/ml for *A. flavus* [70]. Oladiti Olaniyi and Adebowale isolated, screened, and partially purified beta-mannanase from fungi isolated from soil and water samples collected from Ilaje Lake, Nigeria, and found the isolate of *Fusarium solani* to have a mannanase activity of 2.16 IU/mg [71].

In addition to enzyme activity, fungal isolates were also screened for their antioxidant potential. Relatively high to moderate antioxidant activities across the three assays were recorded for isolates F1*: Clonostachys rosea* 655,669 μmol TE/g (ORAC), 12,572 μmol TE/g (DPPH), and 36,019 μmol TE/g (FRAP)*;* F3: *Fusarium avenaceum* 4,488,035 μmol TE/g (ORAC), 22,646 μmol TE/g (DPPH), and 69,325 μmol TE/g (FRAP); F6: *Mucor leutus* 82,144 μmol TE/g (FRAP); F8: *Epicoccum nigrum* 13,700 μmol TE/g (DPPH) and 79,224 μmol TE/g (FRAP); and F9: *Trichoderma koningii* 22,558 μmol TE/g (DPPH) and 25,812 μmol TE/g (FRAP). In general, polar extracts of both cell biomass and liquid culture performed better than the non-polar extracts. Non-polar cell biomass extracts showed little to no antioxidant activity across the three assays. There is little literature where the ORAC activities of crude extracts of fungi have been recorded and displayed as micromolar Trolox equivalent. According to an online database of superfoods [72], the highest ORAC activity was recorded for Dragon's Blood (Croton lechleri) and Astaxanthin Supplements (isolated from microalgae) at 28,971 µmol TE/g and 28,222 µmol TE/g, respectively, which are much lower compared to ORAC activities recorded per gram of extracts in this study. ORAC assay is popularly used to measure the antioxidant activity of plant extracts and superfoods [73–75]. Although they are less used for crude fungal extracts, the addition of DPPH and FRAP assays helped in visualizing the best antioxidant producers in the biobank. Murthy et al. (2011) reported a total antioxidant activity of 20 mg (ascorbic acid equivalent) for the strain of *Mucor* species and 15 mg (ascorbic acid equivalent) for the strain of *Fusarium* [76]. A study by Dong et al. (2014) reported radical scavenging activity for *Fusarium avenaceum* at 13.82 µmol Trolox equivalent/g extract against ABTS and FRAP activity of 81.7 µmol ferrous sulfate/g of extract. Also, the same study reported ABTS and FRAP activity of 14.48 µmol Trolox equivalent/g and 60. 1 µmol ferrous sulfate/g of extract, respectively, for a strain of *Trichoderma* sp. [77]. In another study, three of four compounds purified from a strain of *Epicoccum nigrum* reported DPPH and FRAP activities as 38.43 IC_{50} µg/mL, 88.97 IC_{50} µg/mL, 11.36 IC_{50} µg/mL, and 12.12 µmol ferrous sulfate/g; 6.34 µmol ferrous sulfate/g; and 27.17 µmol ferrous sulfate/g, respectively [78]. While there is evidence in the literature to support the antioxidant capabilities of some of these fungi [77, 78], direct comparison of literature described results to antioxidant activities measured in this study was difficult due to different ways of normalization and use of reference compounds in antioxidant assessment [79].

The secondary metabolite profiles correlated to the antioxidant ability of the isolates. Compounds such as averufin, benzoic acid, toluhydroquinone, and propyl-3,4,5-trihydroxybenzoate all promote antioxidant activity and are found in isolate *Fusarium avenaceum* (F3). Isolate F4: *Fusarium tricinctum* also showed moderate antioxidant activity and its extract contained compounds such as benzoic acid, toluhydroquinone, and orsellinic acid; a phenolic acid; and a terpenoid ophiobolin G. All these classes of compounds have potential antioxidant activity [80–82]. Slightly lower antioxidant activity was recorded for extract isolate *Fusarium cerealis* (F7) which showed the presence of chrysophanol, a natural anthraquinone with antioxidant activity [83].

Another isolate demonstrating good antioxidant activity isolate was F5: *Hypomyces armeniacus* and its extract also showed the presence of compounds like caffeine, orsellinic acid, and cyclopiazonic acid which is an indole tetramic acid known to have antioxidant activity [46]. High antioxidant activity of isolate F8: *Epicoccum nigrum* could be a result of a concerted effort of compounds such as toluhydroquinone, propyl-3,4,5-trihydroxybenzoate, 2',3'-dihydrosorbicillin [84], javanicin and norjavanicin [85, 86], and caffeic acid [87]. This isolate also showed the highest number of metabolites compared to the remaining isolates which could be responsible for its overall bioactivity.

F6: *Mucor luteus* displayed good antioxidant activity which may be attributed to the presence of propyl-3,4,5-trihydroxybenzoate and a quinazoline verrucine A which were detected. Quinazoline compounds have a range of pharmacological activities such as analgesic,

Table 3 Key enzyme-producing strains with antioxidant activity and metabolites

Fungus	Enzymes	Antioxidants	Metabolites	Compounds with potential antioxidant activity
F1 *Clonostachys rosea*	*β*-xylanase and Arabinoxylanase activity	ORAC: Polar cell biomass extract activity at 655,669 *μ*mol TE/g DPPH: Moderate DPPH activity was recorded for polar liquid culture extracts at 12,572 *μ*mol TE/g FRAP: Liquid culture polar extract activity at 36,019 *μ*mol TE/g	Benzoic acid at a relative percentage of 14% followed by 3-Pyridinepropionic acid and cyclo(-Gly-Phe) at 11% and 10% respectively	Chrysophanol, Toluhydroquinone
F2 *Penicillium expansum*	Cellulase and Pectinase activity	ORAC: Polar cell biomass extract activity at 636,889 *μ*mol TE/g	Highest area percentage for Cyclo(-Gly-Phe) at 25% followed by prenitremone A at 19% relative to other identifiable metabolites	Chrysophanol, caffeine
F3 *Fusarium avenaceum*	Galacto-mannanase and Mannanase activity	ORAC: The highest activity was recorded for polar cell biomass extract at 4,488,035 *μ*mol TE/g DPPH: The second-highest activity was recorded for non-polar liquid culture extract at 22,646 *μ*mol TE/g FRAP: Cell biomass extract activity at 69,325 *μ*mol TE/g	Highest peak area was recorded for cyclo(-Leu-Leu) at 35% followed by p-anisaldehyde with a relative peak area of 33% and cyclo(-Gly-Phe) with a moderate peak area percentage of 21% in comparison to other identifiable metabolites	Propyl-3,4,5-trihydroxybenzoate, Toluhydroquinone, Riboflavin, Dehydroculvurin
F9 *Trichoderma koningii*	β-xylanase and Arabinoxylanase activity	DDPH: The third highest activity was recorded for polar liquid culture extract at 22,558 *μ*mol TE/g FRAP: Liquid culture polar extract activity at 25,812 *μ*mol TE/g	Compounds cyclo(-Gly-Phe), canescin, citreoviridin X, and cyclopaldic acid showed relatively comparable peak areas at 20%, 17%, 17%, and 16%, respectively, followed by caffeic acid at 11%	Riboflavin, caffeine, Propyl-3,4,5-trihydroxybenzoate, Diaportinol

anti-inflammatory, anti-convulsant, sedative-hypnotic, anti-histaminic, and anti-hypertensive being a few of these activities and as such could have contributed to the antioxidant effect of these isolates [88].

F9: *Trichoderma koningii* showed the presence of caffeic acid, caffeine, an isocoumarin diaportinol, jasmonic acid, riboflavin, and propyl-3,4,5-trihydroxybenazoate, all of which could have resulted in the high antioxidant activity of this isolate [88–90].

From the LC–MS analysis of fungal extracts reported here, an important class of compounds identified in isolates were cyclic peptides such as the simple cyclic dipeptide cyclo(-Gly-Phe) found in all the extracts followed by other dipeptides cyclo(-Leu-Leu) and cyclo(-Gly-Pro) also present in isolates' profiles. The highest number of cyclic dipeptides was found in isolate F8: *Epicoccum nigrum*. Other cyclic peptides include apicidin found in isolate F3: *Fusarium avenaceum*; 2,5-diketopiperazines such as roquefortine C found in isolate F1: *Clonostachys rosea*; and rugulosuvine found in isolate F9: *Trichoderma koningii*.

Cyclization of peptides creates desirable structural and functional features that are critical for their use as pharmaceutical agents [91]. Isolated cyclic peptides have been used as therapeutic agents. For example, Cyclosporin A, a cyclic non-ribosomal peptide of eleven amino acids, isolated from fungus *Tolypocladium inflatum* has potent immunosuppressive activity and prevents rejection of kidney, heart, and liver after transplants [92]. Some cyclic peptides identified in fungi such as penicillin, ergopeptides, and cephalosporins have gained high success in the pharmaceutical industry [93–95]. Simple diketopiperazines are a type of cyclic dipeptides that may be detected in the fermentation of many fungi and reportedly have extensive antimicrobial, phototoxic, cytotoxic, nematicidal, radical scavenging, antioxidant, and antiviral activities [96–98].

To summarize, isolates of *Penicillium*, *Trichoderma*, *Fusarium*, and *Clonostachys* were the most prominent producers of either select or a cocktail of enzymes effectively degrading substrates found in plant biomass (Table 3). These strains also showed ORAC, DPPH, and FRAP activity making them potential candidates as a source of natural microbial antioxidants. Table 3 also shows compounds found in the extracts that could correlate to the antioxidant activity of these fungi. Additionally, an LCMS protocol was developed which is simple and rapid and allows identification of key metabolites and mycotoxins present in fungal cultures. This protocol can be applied to future fermentations optimized for enzyme production.

Conclusion

The methods presented here for the creation and screening of a fungal biobank for enzyme, antioxidant, and metabolite production can be adapted to any fungal biobank for the discovery of new industrially relevant compounds and enzymes. Here, we have reported the isolation and creation of a new fungal biobank from Irish habitats, we have identified the best strains for enzyme activity, and we have also reported antioxidant activity and secondary metabolite production from these enzyme producers (Table 3). The LCMS protocol presented here is a simple and rapid method to analyze and identify key metabolites and mycotoxins present in fungal cultures.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44314-024-00015-0.

Additional file 1: Table S1. List of 23 fungal isolates chosen for investigation studies. Tables S2–S10. Fungal metabolites identified for isolates F1–F9 using molecular features by LC-MS. Table S11. List of secondary metabolites of fungal isolates. Figures S1–S6 display enzyme activities for cellulase, pectinase, xylanase, arabinoxylanase, galacto-mannanase, and mannanase activity in U/mg of protein for fungal isolate.

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Authors' contributions
Conceptualization, J.K. and C.C.; methodology, J.K.; validation, J.K., C.C. and P.M.; investigation, J.K.; resources, J.K.; data curation, J.K.; writing—original draft preparation, J.K.; writing—review and editing, C.C and P.M.; visualization, C.C and P.M.; supervision, C.C and P.M.; project administration, J.K.; funding acquisition, C.C and P.M. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University (KY2022-175). All the research participants provided informed consent and were kept anonymous.

Consent for publication
All authors approved the final manuscript and the submission to the journal.

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