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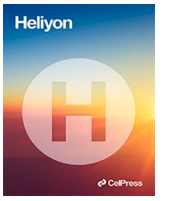

Research article

Multigene phylogeny, bioactive properties, enzymatic and dye decolorization potential of selected marine fungi from brown algae and sponges of Mauritius

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ABSTRACT

Marine fungi represent an important proportion of the microbial diversity in the oceans. They are attractive candidates for biotechnological purposes and industrial applications. Despite an increasing interest in mycology, marine fungi associated with sponges and algae have been poorly studied in Mauritius. The objectives of this study were to: 1) use multigene phylogenetic analyses to identify isolated marine fungi; 2) determine the differences in the antimicrobial and antioxidant properties of the fungal extracts; and 3) assess their enzyme activities and dye decolorization potential. Five fungal isolates *viz Aspergillus chevalieri*, *Aspergillus iizukae, Aspergillus ochraceus*, *Exserohilum rostratum* and *Biatriospora* sp. were identified based on phylogenetic analyses. There was no significant difference in the antimicrobial properties of the liquid and solid media extracts unlike the antioxidant properties ($p < 0.05$). The solid media extract of *Aspergillus chevalieri* (F2-SF) had a minimum inhibitory concentration of 0.156 mg/ml against *Staphylococcus aureus* while *Aspergillus ochraceus* (F25-SF) had a minimum inhibitory concentration of 0.313 and 2.5 mg/ml against *Enterococcus faecalis* and *Salmonella typhi*. The solid media extract of *Biatriospora* sp. (F34-SF) had a minimum inhibitory concentration of 0.195 and 1.563 mg/ml against *Bacillus cereus*, *Escherichia coli* and *Enterobacter cloacae*. An IC_{50} of 78.92 ± 4.71 μg/ml in the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, ferric reducing antioxidant power (FRAP) value of 11.17 ± 0.20 mM Fe^{2+}/g dry weight extract (DWE) and total phenolic content 360.35 ± 10.31 mg GAE/g DWE was obtained with the solid media extract of *Aspergillus chevalieri* (F2-SF). *Aspergillus ochraceus* (F25-SF) and *Biatriospora* sp. (F34-SF) solid media extracts showed lower IC_{50} values in the DPPH assay and higher total phenolic content as compared to the liquid media extracts. *Aspergillus chevalieri* was a good producer of the enzymes DNAse and lipase and had maximum percentage dye decolorization of 79.40 ± 17.72% on Congo red. An enzymatic index ≥ 2 was found for the DNAse and lipase and the maximum percentage dye decolorization of 87.18 ±

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3.80% was observed with *Aspergillus ochraceus* on Methylene blue. Regarding *Biatriospora* sp., it was a moderate producer of the three enzymes amylase, DNAse and protease and had a maximum dye decolorization potential of 56.29 ± 6.51% on Crystal violet. This study demonstrates that Mauritian marine fungi possess good bioactive properties, enzymatic and dye decolorization potentials, that can potentially be considered for use in pharmaceutical and industrial applications.

1. Introduction

Marine fungi are ubiquitous heterotrophic eukaryotes that are associated with sediments, sand, mangroves, driftwoods, algae and sea grass, sponges, ascidians, corals and sea animals [1–3]. According to Amend et al. [4], approximately 1100 species have been recovered from the oceans, indicating the lack of research on these. Marine fungi have long been classified as obligate or facultative, based on their ecological niche [5]. Nevertheless, this definition has been reviewed and a broader definition was proposed by Pang et al. [6]. A marine fungus is "any fungus that is recovered repeatedly from marine habitats because: 1) it is able to grow and/or sporulate (on substrata) in marine environments; 2) it forms symbiotic relationships with other marine organisms; or 3) it is shown to adapt and evolve at the genetic level or be metabolically active in marine environments".

The majority of fungi isolated from marine environments belong to the phylum Ascomycota or Basidiomycota. Morphological characters, to assign putative identity, are not reliable as some fungi fail to sporulate under culture conditions [7,8]. Microscopy and DNA-based methods have helped in the proper identification of the species residing in a particular habitat. Amplicon sequencing, based on the fungal internal transcribed spacer (ITS) rDNA regions, has allowed successful identification of a wide range of fungal species [8–10]. Nevertheless, only 75% of these species can be identified accurately using the single barcoding ITS regions. A secondary barcode region like the translation elongation factor (TEF) is often used to improve identification [8,11]. In the *Aspergillus*, *Penicillium* and *Talaromyces* genus, the ITS, large subunit (LSU) loci and some protein-coding genes such as largest subunit of RNA polymerase (RPB1), beta-tubulin (TUB2) and partial calmodulin (CaM) are more effective [8,12].

The oceans contain a vast majority of organisms, which represent unique mines of natural bioactive compounds. Numerous research oriented on the marine micro-organisms, such as bacteria and fungi, have been carried out and interesting results were found. The latter have the advantage of being cultivated in the laboratory to produce large amounts of secondary metabolites [13]. Marine fungi are important colonisers of the oceans and represent a prolific source of secondary metabolites that have antimicrobial, antioxidant, anti-cancer, anti-inflammatory, antiviral, anti-diabetic and neuroprotective properties [14–16]. Compounds isolated from marine fungi include alkaloids, polyketides, coumarins, polyphenols, xanthones, terpenoids, steroids and peptides [13,17–19].

Bioactive compounds are of great interest as they protect the human body from harmful microorganisms and high levels of free radicals and reactive oxygen species (ROS) that lead to diseases [14–16]. Bacterial infections can rapidly spread throughout the population via airborne routes, vectors or by direct contact. This results in severe illness and death, impacting on the health sector and the economy [20]. The WHO has declared that antimicrobial resistance is among the top 10 global health threats [20] and the search for antimicrobial compounds that kill multidrug-resistant bacteria is of uttermost importance. Fungi have been considered as producers of antimicrobial compounds since the discovery of cephalosporin by Guiseppe Brotzu in 1948. The beta-lactam antimicrobial, cephalosporin C, was isolated from the fungus *Cephalosporium acremonium*, obtained from a sewage outfall of the Sardinian coast [21]. Reactive oxygen species (ROS) produced during normal cellular processes [22,23] can be harmful at high concentrations and affect many cellular functions leading to degenerative diseases such as cancer, cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress [24]. Natural antioxidants such as phenolic compounds, alkaloids, anthraquinones, xanthones and carotenoids from fungi can be potent radical scavengers and effective inhibitors of lipid peroxidation, thereby protecting cells against oxidative stress [25–27].

In general, fungi are ubiquitous heterotrophic eukaryotes which actively secrete enzymes into their environment to digest their food [28]. Being the primary decomposers in the marine ecosystem, they are known to produce hydrolytic and oxidative enzymes with amylase, cellulase, chitinase, keratinase, inulinase, ligninase, lipase, nuclease, phytase, protease and xylanase activities. This characteristic is very attractive as they can be used in the textile, biofuel, paper, food, animal feed, pharmaceutical and cosmetic industry [29,30]. Through their extracellular enzymatic system, marine fungi can also degrade many toxic and mutagenic synthetic dyes that are used in industry [31]. Those aromatic compounds reduce the dissolved oxygen and are poisonous to aquatic life. Mycoremediation is effective, eco-friendly and economical as compared to traditional methods that are costly and sometimes result in accumulation of secondary materials [32,33].

The objectives of this study were to: 1) identify marine fungi isolated from sponges and brown algae of Mauritius; 2) determine the significant difference between the bioactive properties of the liquid media and solid media culture extracts; and 3) assess the production of extracellular hydrolytic enzymes by the fungi and their dye decolorization potential.

2. Materials and methods

2.1. Multigene phylogeny of selected fungi

Five fungi, which were obtained from the previous work of Wong Chin et al. [34] were selected for multigene phylogenetic

analyses. In the previous work, these isolates were identified based on ITS rDNA sequences and phylogenetic analyses. They were *Aspergillus cristatus* (F2), *Aspergillus ochraceus* (F25), *Exserohilum rostratum* (F33), *Chromocleista* sp. (F34) and *Aspergillus flavipes* (F37) [34].

2.1.1. DNA extraction, polymerase chain reaction amplification and sequencing

Total genomic DNA was extracted from fresh mycelium, growing on PDA, using the Norgen Biotek Plant/Fungi DNA isolation kit (Norgen Biotek Corp, Cat. 26200), according to the manufacturer's instructions. Polymerase chain reaction (PCR) of the DNA was then carried out using the primers and the conditions listed in Table 1. The final volume of the PCR reaction was 25 μl and consisted of 2 μl of DNA template, 1 μl of forward and reverse primer, 12.5 μl of 2*X* PCR Mastermix (Thermo Scientific™) and 8.5 μl of nuclease-free water. The amplified PCR products were sent to Macrogen Europe BV (Amsterdam, Netherlands) for sequencing.

2.1.2. Multigene phylogenetic analyses

Protein-coding and ribosomal gene sequences were analysed to construct a multigene phylogeny. Single gene alignment was performed in MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/) and manually edited in Bioedit. Further analyses were performed using the Maximum likelihood method RAxML-HPC2 on XSEDE with 1000 bootstrap replicates using the GTRGAMMA model of nucleotide substitution. The maximum likelihood phylogenetic tree generated was viewed in FigTree [35]. The sequences obtained in this study were deposited in GenBank under accession number OQ754174 – OQ941652.

2.2. Bioactive properties of selected fungi

Three fungi were selected for further studies; namely *Aspergillus chevalieri* (F2), *Aspergillus ochraceus* (F25) and *Biatriospora* sp. (F34). The liquid media extracts showed antimicrobial properties [34].

2.2.1. Solid media cultivation of selected marine fungi

In addition to the liquid media cultivation of the three selected marine fungi (reported previously by Wong Chin et al. [34]), solid media cultivation was also done. Five plugs of the fungus were used to inoculate the rice media (100 g rice, 100 ml seawater). The fungus was allowed to grow under static conditions, at room temperature, for 30 days [36].

2.2.2. Extraction of solid media secondary metabolites

After 30 days, the rice culture was chopped into small pieces with a sterile spatula and 250 ml of ethyl acetate (Loba Chemie, India) was added to each flask. The ethyl acetate was collected after 48 h and evaporated at 40 °C using a rotary evaporator (Heidolph, Germany). The crude extract was re-suspended in dimethyl sulfoxide (DMSO) (Loba Chemie, India) to make a stock solution of 40 mg/ml [3].

2.2.3. Antimicrobial property of solid media fungal extract

Disc diffusion assay and broth microdilution method were carried out with the solid media fungal extracts, as described previously [34]. The same bacteria were used, namely, the three Gram-positive bacteria, *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213) and the three Gram-negative bacteria, *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC 13047). Chloramphenicol (1 mg/ml) (Sigma-Aldrich, Germany) was used as a positive control. The liquid media mycelium extracts were labelled as F2M, F25M and F34M, while the solid media extracts were labelled as F2-SF, F25-SF and F34-SF.

2.2.4. Antioxidant activity of fungal extracts

2.2.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. For the DPPH assay, 1 ml of extract (of varying concentration) and 2 ml of 100 μM DPPH (Sigma-Aldrich, Germany) were incubated for 30 min at room temperature in the dark. The absorbance was then read at 517 nm using a spectrometer (Jenway-7305 spectrometer UV–visible, UK). The negative control contained the extract vehicle and the positive control was ascorbic acid (Sigma-Aldrich, Germany). The percentage of radical scavenging activity was calculated according to equation (1). A concentration response curve was generated, and the IC_{50} value was determined. All the experiments were performed in triplicates [37].

Table 1
Details of genes/loci, PCR primers and conditions used.

Gene/Loci	PCR primers	PCR conditions
ITS	ITS5, ITS4	95 °C: 3 min, 35 cycles (95 °C: 1 min, 52 °C: 50 s, 72 °C: 1 min), 72 °C: 10 min
LSU	LROR, LR5	95 °C: 3 min, 35 cycles (95 °C: 1 min, 54 °C: 50 s, 72 °C: 1 min), 72 °C: 10 min
TUB2	Bt2a, Bt2b	
CaM	CAL737R, CAL228F	
ACT	ACT512F, ACT783R	

$$\% \text{ Scavenging activity} = \left(\frac{Absorbance\ negative\ control - Absorbance\ extract}{Absorbance\ negative\ control} \right) * 100 \quad (1)$$

2.2.4.2. Ferric reducing antioxidant power assay. The FRAP assay was performed by mixing 1.5 ml of the FRAP reagent, 50 μl of extract and 150 μl of distilled water. The FRAP reagent was prepared by mixing 20 ml of 10 mM TPTZ in 40 mM HCl, 20 ml of 20 mM ferric chloride and 200 ml of 0.25 M sodium acetate buffer (pH 3.6) (Sigma-Aldrich, Germany). After incubation at 37 °C for 4 min, the absorbance was read at 593 nm against a blank. A standard curve of ferrous sulfate (Sigma-Aldrich, Germany) was used to express the results in mmol Fe2+/g dry weight extract (DWE) [25].

2.2.4.3. Determination of total phenolic content. The total phenolic content was determined using the Folin-Ciocalteu assay. In a test-tube, 0.25 ml of extract, 3.50 ml distilled water and 0.25 ml of Folin-Ciocalteu reagent (Loba Chemie, India) were mixed. After 5 min, 1 ml of 20% sodium carbonate (Sigma-Aldrich, Germany) was added and the mixture was incubated at 40 °C for 40 min. The absorbance was then read at 685 nm against a blank. A standard curve of gallic acid (Sigma-Aldrich, Germany) was used and the results were expressed as mg gallic acid equivalent (GAE)/g DWE [25].

2.3. Extracellular enzyme production

The extracellular enzyme production, of the three isolates F2, F25 and F34, was assessed according to Jagannat et al. [38] and de Oliveira Amaral et al. [39].

2.3.1. Amylase

The amylase production was assessed by growing the fungus on Starch M-protein agar (Himedia®). The plates were incubated for 7 days. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The zone of clearance around the colony indicated amylase activity.

2.3.2. DNAse

DNAse activity was assessed by growing the fungus on DNAse agar (Oxoid™). After incubation of 7 days, the plate was flooded with 1 N HCl. Clear zone around the colony in an otherwise opaque medium indicated degradation of DNA.

2.3.3. Lipase

The lipase production was assessed by growing the fungus on peptone agar (10 g peptone (Himedia®), 5 g NaCl, 0.1 g $CaCl_2$ (Sigma-Aldrich, Germany), 16 g agar (Himedia®), 1000 ml distilled water) supplemented with 1% Tween 20 (Sigma-Aldrich, Germany). The Tween 20 was sterilized separately and added to the media before pouring. A precipitate around the colony, after 7-day incubation, indicated the presence of lipase.

2.3.4. Cellulase

The cellulase production was assessed by growing the fungus on glucose yeast extract agar medium (1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar (Himedia®), 1000 ml distilled water) supplemented with 0.5% methyl cellulose (Sigma-Aldrich, Germany). After incubation, the plates were flooded with 0.1% aqueous Congo red solution and de-stained with 1 M NaCl for 15 min. A clear zone around the colony indicated cellulase production.

2.3.5. Gelatinase

The gelatinase production was assessed by growing the fungus in glucose yeast extract peptone broth (Himedia®) supplemented with 1% gelatin (Sigma-Aldrich, Germany). After incubation, the tubes were placed at 4 °C overnight. Presence of gelatinase was confirmed by the liquefaction of the broth.

2.3.6. Protease

The protease production was assessed by growing the fungus on glucose yeast extract agar (Himedia®) supplemented with 2% skim milk. After incubation, a clear zone around the colony indicated protease activity.

2.3.7. Urease

Urease production was assessed by growing the fungus on Christensen's Urea agar (Himedia®). Development of a pink colour indicated urease activity.

The enzymatic index (EI) was then calculated according to equation 2.

$$EI = \frac{R}{r} \quad (2)$$

where R is the diameter of the clear zone and r is the diameter of the colony [39].

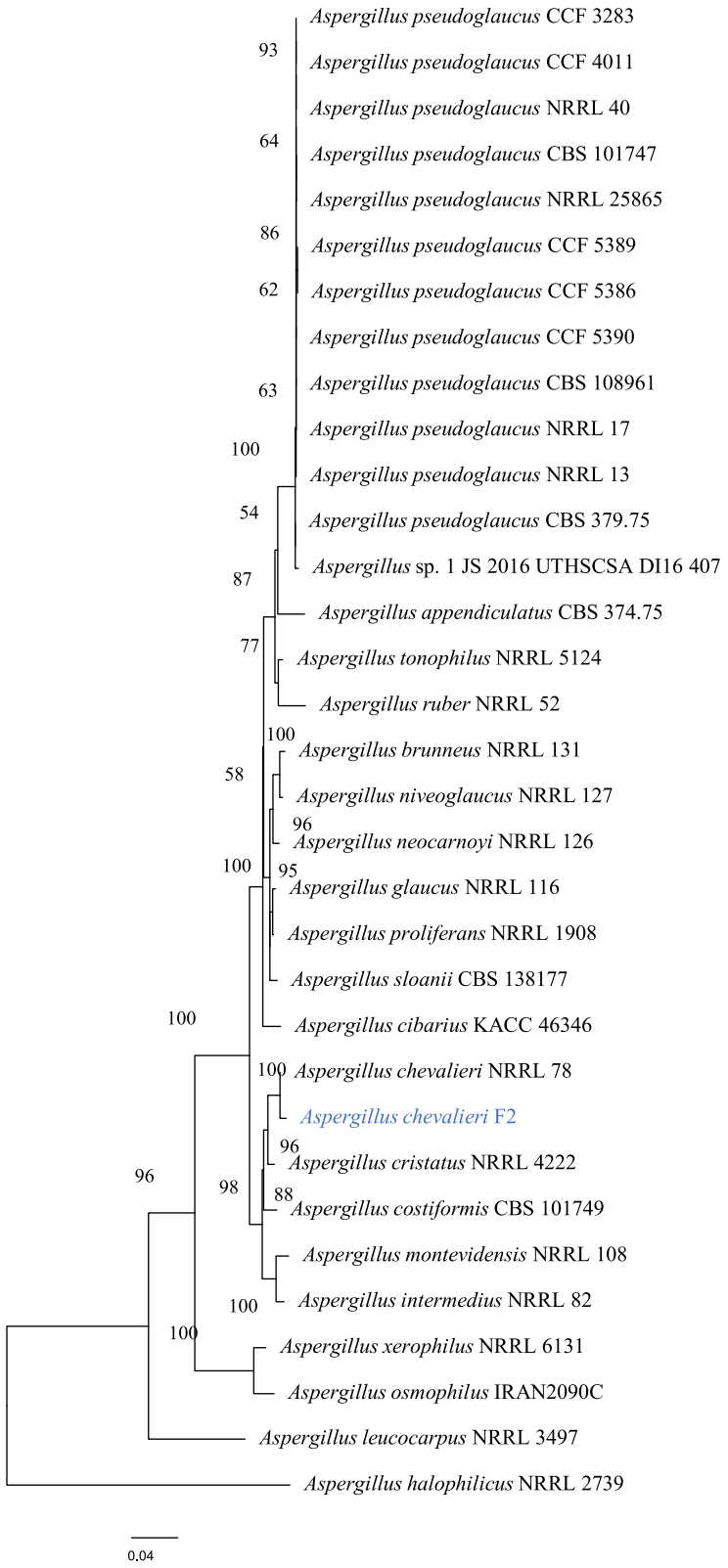

Fig. 1. RAxML tree based on analysis of a combined LSU, TUB2, CaM and ITS sequence dataset. Bootstrap support values for maximum likelihood higher than 50% are given above each branch. The tree is rooted to *Aspergillus halophilicus*.

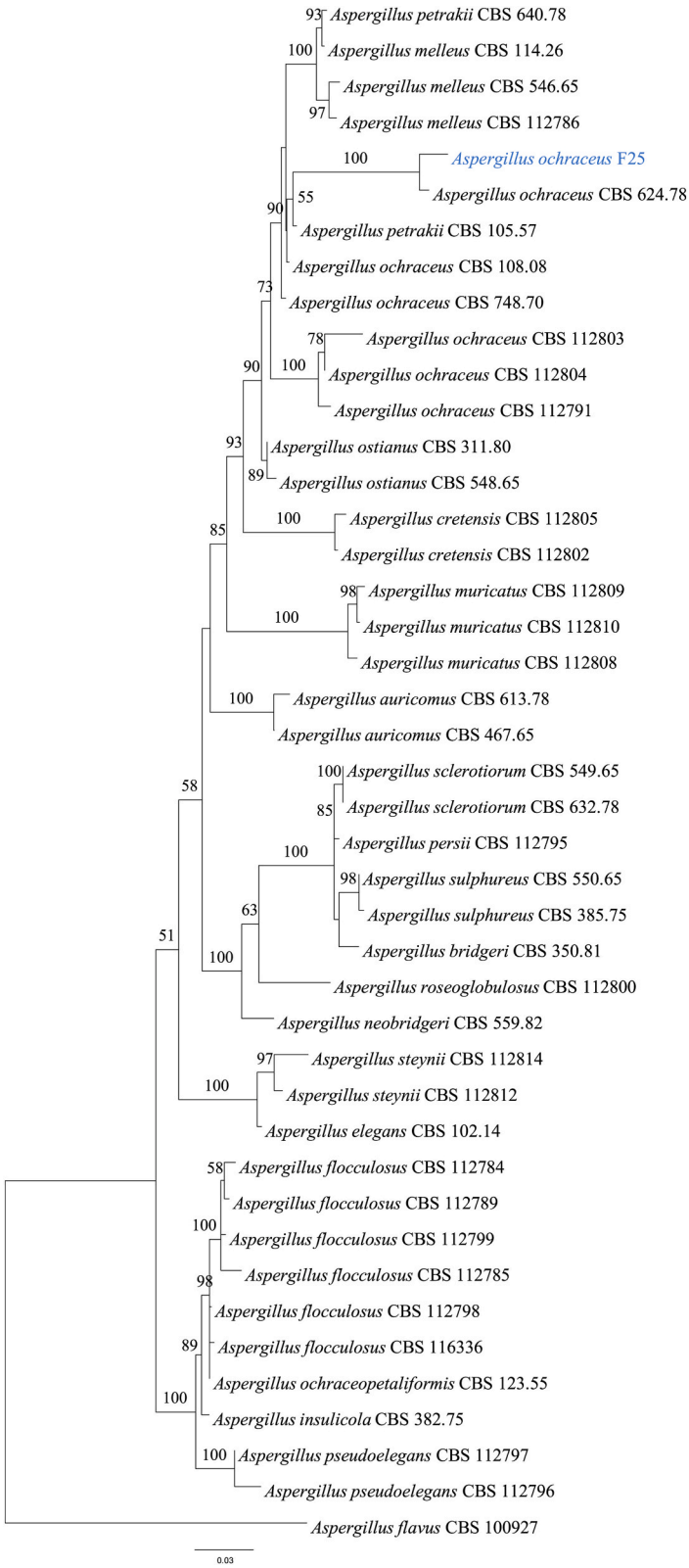

Fig. 2. RAxML tree based on analysis of a combined LSU, TUB2 and ITS sequence dataset. Bootstrap support values for maximum likelihood higher than 50% are given above each branch. The tree is rooted to *Aspergillus flavus*.

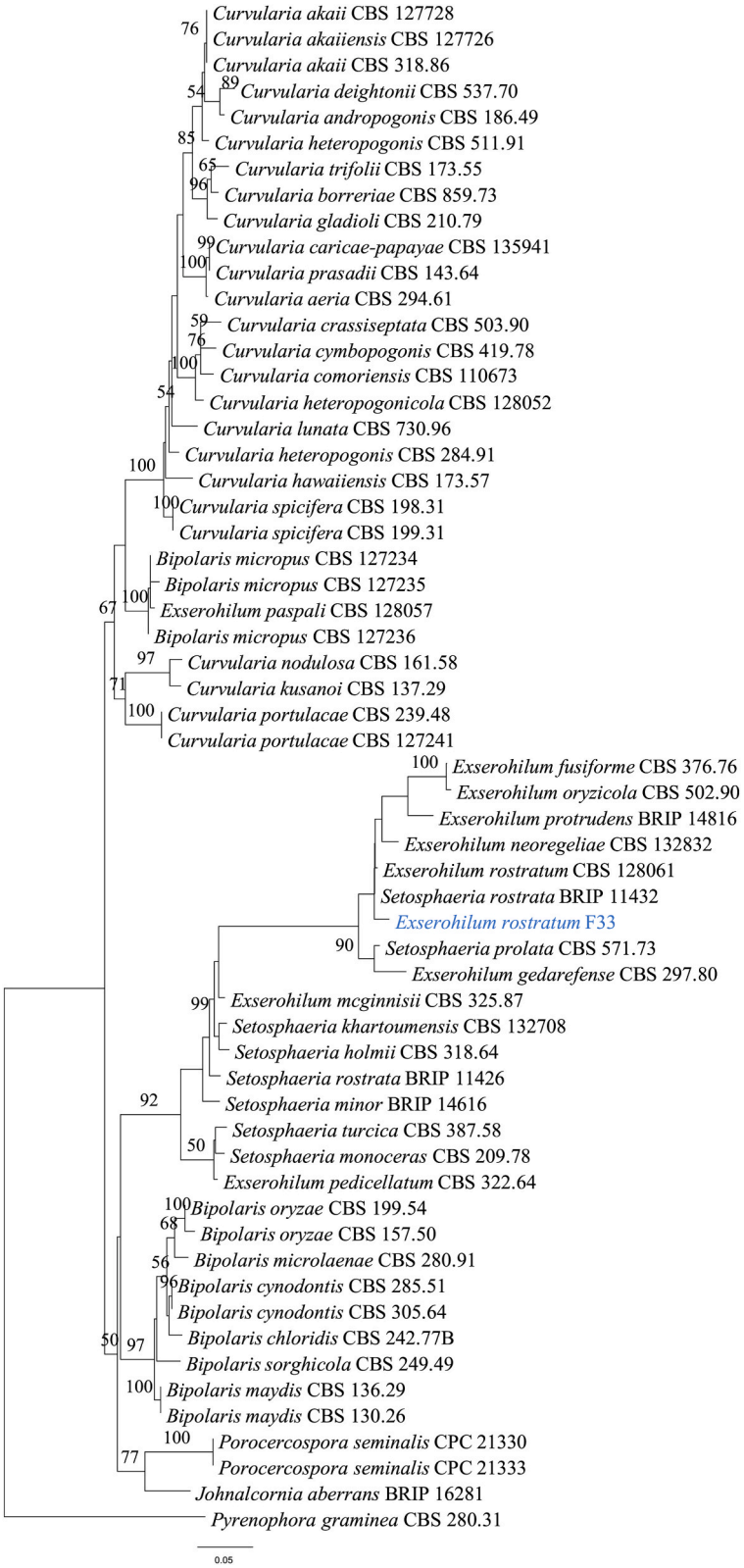

Fig. 3. RAxML tree based on analysis of a combined LSU, ACT, CaM and ITS sequence dataset. Bootstrap support values for maximum likelihood higher than 50% are given above each branch. The tree is rooted to *Pyrenophora graminea*.

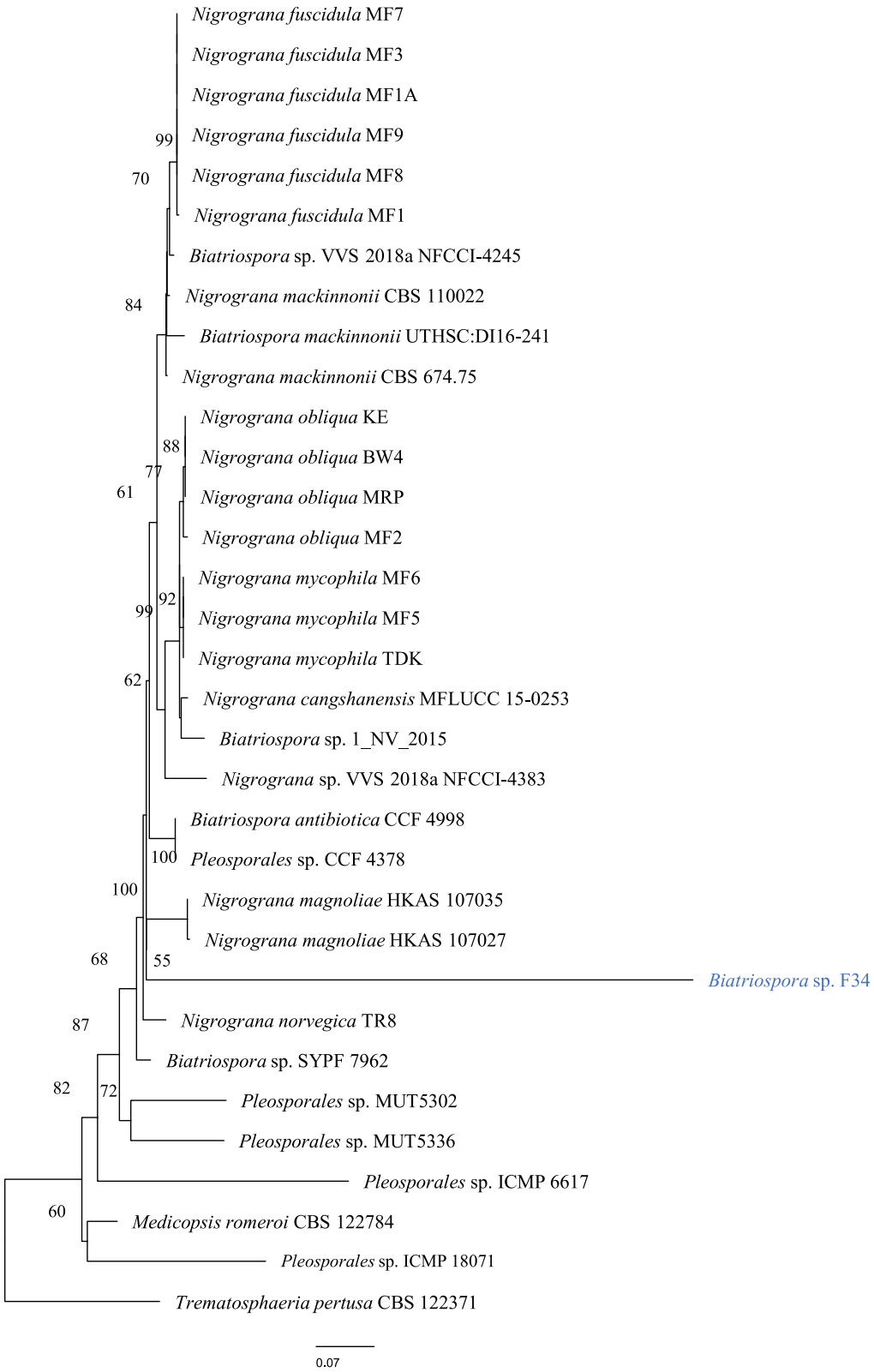

Fig. 4. RAxML tree based on analysis of a combined LSU, TUB2, ACT and ITS sequence dataset. Bootstrap support values for maximum likelihood higher than 50% are given above each branch. The tree is rooted to *Trematosphaeria pertusa* and *Medicopsis remeroi*.

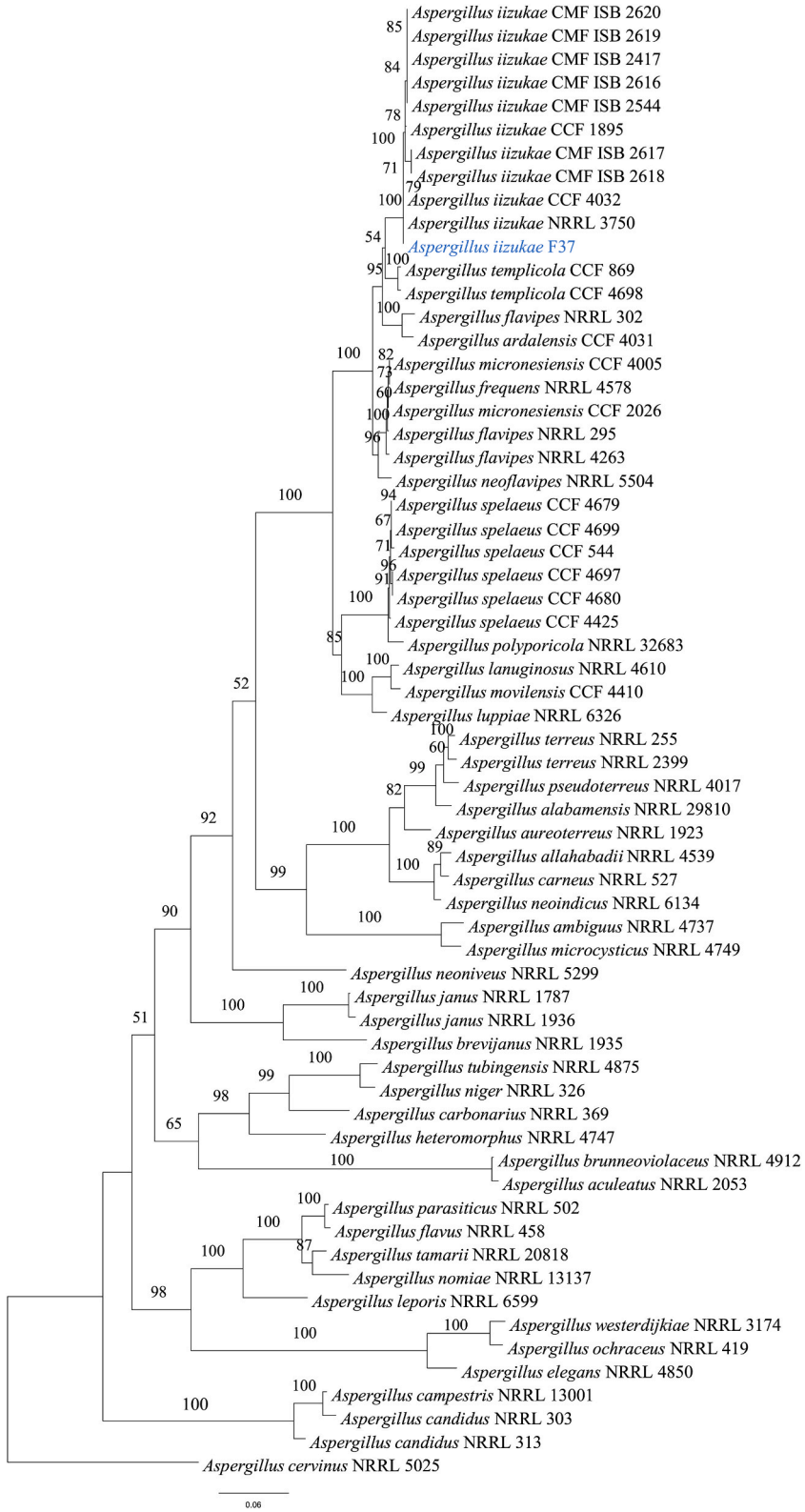

Fig. 5. RAxML tree based on analysis of a combined LSU, TUB2 and ITS sequence dataset. Bootstrap support values for maximum likelihood higher than 50% are given above each branch. The tree is rooted to *Aspergillus cervinus*.

2.4. Dye decolorization of marine fungi

A plug of actively growing mycelium (3 mm × 3 mm) was inoculated into 25 ml of seawater potato dextrose broth (PDB) (Himedia®), supplemented with 0.01% of one of the following dyes: Congo red, Malachite green, Methylene blue and Crystal violet (Sigma-Aldrich, Germany). The culture was allowed to grow at room temperature for 10 days under shaking condition. After 10 days, the culture filtrates were subjected to spectrophotometric analysis. The absorbance was read at the following wavelengths: Congo red- 497 nm, Malachite green- 620 nm, Methylene blue and Crystal violet- 590 nm. The percentage dye decolorization of the marine fungi was calculated using equation (3) [40].

$$\%\ \text{Dye decolorization} = \frac{Abs\ (control) - Abs\ (inoculated)}{Abs\ (control)}\ x\ 100 \tag{3}$$

2.5. Statistical analysis

Results were analysed using a two-way ANOVA in Minitab 17 to determine if there was a significant difference between the antimicrobial and antioxidant properties of liquid media cultivation and the solid media cultivation fungal extracts, as well as the dye decolorization potential of the selected fungi. One-way ANOVA followed by Tukey post hoc test was also used to determine if there was a significant difference between the extracts.

3. Results

3.1. Multigene phylogeny

For *Aspergillus chevalieri* (F2): The combined LSU, TUB2, CaM and ITS sequence dataset comprised 33 specimens with *Aspergillus halophilicus* NRRL 2739 as outgroup. There were 481 distinct alignment patterns. The RAxML tree is shown in Fig. 1 and the final ML optimization likelihood value is −6914.1478. Rate parameters: A-C: 0.8493, A-G: 4.1007, A-T: 1.6883, C-G: 0.7085, C-T: 6.0925, G-T: 1.0000; Base frequencies: A: 0.2285, C: 0.2605, G: 0.2677, T: 0.2433.

Isolate F2, which was identified as *Aspergillus cristatus*, was closely related to *Aspergillus chevalieri* NRRL 78 species with high bootstrap value of 100%. F2 was therefore identified as *Aspergillus chevalieri*.

For *Aspergillus ochraceus* (F25): The combined LSU, TUB2 and ITS sequence dataset comprised 43 specimens with *Aspergillus flavus* CBS 100927 as outgroup. There were 635 distinct alignment patterns. The RAxML tree is shown in Fig. 2 and the final ML optimization likelihood value is −8894.3778. Rate parameters: A-C: 1.1556, A-G: 1.7949, A-T: 0.8851, C-G: 0.5865, C-T: 2.7531, G-T: 1.0000; Base frequencies: A: 0.2137, C: 0.2759, G: 0.2726, T: 0.23789.

Isolate F25, which was identified as *Aspergillus ochraceus*, was closely related to *Aspergillus ochraceus* CBS 62478 species with high bootstrap value of 100%. It was thus confirmed that F25 was an *Aspergillus ochraceus* isolate.

For *Exserohilum rostratum* (F33): The combined LSU, ACT, CaM and ITS sequence dataset comprised 59 specimens with *Pyrenophora graminea* CBS 280.31 as outgroup. There were 734 distinct alignment patterns. The RAxML tree is shown in Fig. 3 and the final ML optimization likelihood value is −11418.0439. Rate parameters: A-C: 0.9209, A-G: 1.3923, A-T: 0.8739, C-G: 1.1197, C-T: 3.6159, G-T: 1.0000; Base frequencies: A: 0.2398, C: 0.2412, G: 0.2724, T: 0.2467.

Isolate F33, which was identified as *Exserohilum rostratum*, was closely related to *Exserohilum rostratum* CBS 128061 and *Setosphaeria rostrata* BRIP 11432 species with bootstrap value of 90%. The identity of F33 was confirmed to be *Exserohilum rostratum*.

For *Biatriospora* sp. (F34): The combined LSU, TUB2, ACT and ITS sequence dataset comprised 37 specimens with *Trematosphaeria pertusa* CBS 122371 and*Medicopsis romeroi* CBS 122784 as outgroups. There were 668 alignment patterns. The RAxML tree is shown in Fig. 4 and the final ML optimization likelihood value is −9328.0873. Rate parameters: A-C: 1.5599, A-G: 2.9936, A-T: 1.0632, C-G: 1.0657, C-T: 6.1623, G-T: 1.0000; Base frequencies: A: 0.2443, C: 0.2358, G: 0.2747, T: 0.2453.

Isolate F34, which was identified as *Chromocleista* sp., was closely related to many species of *Biatriospora* and *Nigrograna* with bootstrap value of 55%. The concatenated dataset of the LSU and ITS sequences showed isolate F34 was closely related to *Biatriospora antibiotica* CCF 4998 and was therefore identified as *Biatriospora* sp.

For *Aspergillus iizukae* (F37): The combined LSU, TUB2 and ITS sequence dataset comprised 63 specimens with *Aspergillus cervinus* NRRL 5025 as outgroup. There were 675 alignment patterns. The RAxML tree is shown in Fig. 5 and the final ML optimization likelihood value is −13616.427. Rate parameters: A-C: 1.3395, A-G: 3.4927, A-T: 1.6358, C-G: 0.6795, C-T: 5.3529, G-T: 1.0000; Base frequencies: A: 0.2087, C: 0.2872, G: 0.2808, T: 0.2233.

Isolate F37, which was identified as *Aspergillus flavipes*, was closely related to many *Aspergillus iizukae* species with bootstrap value of 54%. F37 was therefore identified as *Aspergillus iizukae*.

3.2. Bioactive properties of selected fungi

3.2.1. Antimicrobial property of solid media fungal extract

The three extracts from the solid media cultivation showed antimicrobial activity against the three Gram-positive bacteria, *B. cereus*, *E. faecalis* and *S. aureus* and the three Gram-negative bacteria, *E. cloacae*, *E. coli* and *S. typhimurium*, included in the study. The mean zone of inhibition (ZOI), which indicates the area of media where bacteria growth was inhibited, ranged from 6.7 ± 0.58 mm to

18 ± 0.58 mm, as shown in Fig. 6. It was the extract of *A. chevalieri* (F2-SF) that had the highest antimicrobial activity against *Bacillus cereus*, with ZOI of 18 ± 0.58 mm. The antimicrobial activity of the different extracts was significantly different as $p < 0.05$. The pairwise comparisons showed no significant difference between the ZOI of liquid media mycelium extracts (reported in Wong Chin et al. [34]) and the ZOI of solid media cultivation extracts. There was a significant difference between the ZOI of the extracts and the positive control ($p < 0.05$).

The minimum inhibitory concentration (MIC) is the lowest concentration of the extract that was able to prevent bacterial growth. The three replicates were consistent. MIC values ranged from 0.156 mg/ml to 5 mg/ml, as shown in Fig. 7. It was the extract of *A. chevalieri* (F2-SF) that had the lowest MIC of 0.156 mg/ml against the bacteria *Staphylococcus aureus*. The pairwise comparisons showed no significant difference between the MIC of liquid media mycelium extracts (reported previously [34]) and the MIC obtained with solid media cultivation extracts.

3.2.2. Antioxidant potential of the fungal extracts

All the extracts showed some antioxidant potential and had varying phenolic content, as shown in Table 2. The extracts obtained from the solid media cultivation showed lower IC_{50} values, in the DPPH scavenging assay. The IC_{50} value represents the concentration of the extract that is required to scavenge 50% of the DPPH radicals. Isolate *A. chevalieri* (F2-SF), grown on the solid media, showed high antioxidant potential with IC_{50} value of 78.92 ± 4.71 μg/ml in the DPPH assay and FRAP value of 11.17 ± 0.20 mM Fe^{2+}/g DWE.

The total phenolic content (TPC) ranged from 32.05 ± 0.16 mg GAE/g DWE to 360.35 ± 10.31 mg GAE/g DWE. Overall, a higher TPC was observed in the solid media extracts. *Aspergillus chevalieri* extract (F2-SF) had the highest TPC.

There was a significant difference between the free radical scavenging activity of the extracts ($p < 0.05$). The TPC of the extracts were also different ($p < 0.05$). The pairwise comparisons indicated that the antioxidant properties of the liquid media mycelium extracts were significantly different compared to the solid media extracts.

3.3. Extracellular enzyme production

The three marine fungi were classified according to the enzyme index (EI), reported in Table 3. *Aspergillus chevalieri* (F2) was a good producer of DNAse and lipase as EI ≥ 2. This isolate produced only the three enzymes amylase, DNAse and lipase. *Aspergillus ochraceus* (F25) was the only isolate which was a moderate producer (EI > 1 and < 2) of the seven enzymes investigated. Moreover, it was the only isolate which was able to produce cellulase.

3.4. Dye decolorization

The three isolates showed significant dye decolorization abilities ($p < 0.05$), as shown in Fig. 8. It was *A. ochraceus* (F25) that had the highest percentage dye decolorization of 87.18 ± 3.80% on Methylene blue. *A. chevalieri* (F2) and *Biatriospora* sp. (F34) had highest percentage dye decolorization of 79.40 ± 17.72% and 56.29 ± 6.51% on Congo red and Crystal violet, respectively.

4. Discussion

The internal transcribed spacer regions (ITS) are the standard fungal barcode, used in fungal diversity studies [41]. However, for some genera like *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium* and *Fusarium*, species level identification, using only the ITS regions, has been problematic [9]. Additional DNA markers for *Aspergillus* such as translation elongation factor (TEF1- α), calmodulin (CaM),

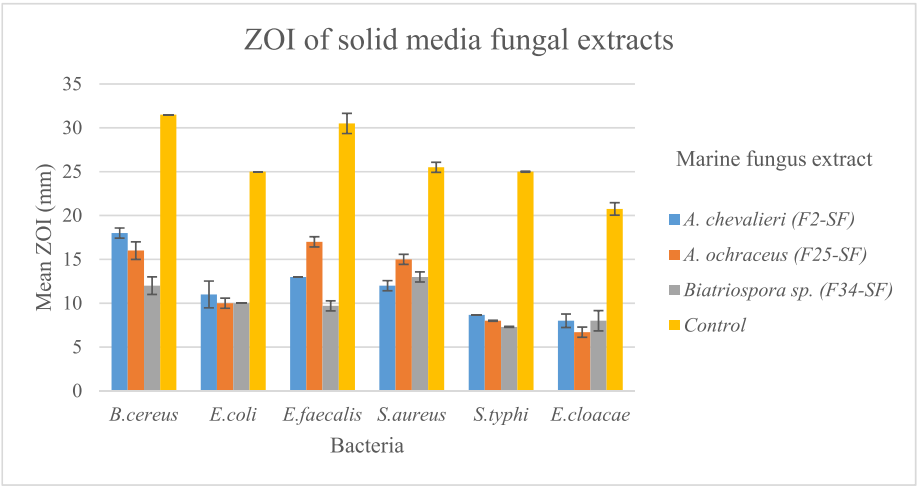

Fig. 6. Mean zone of inhibition of the solid media fungal extracts.

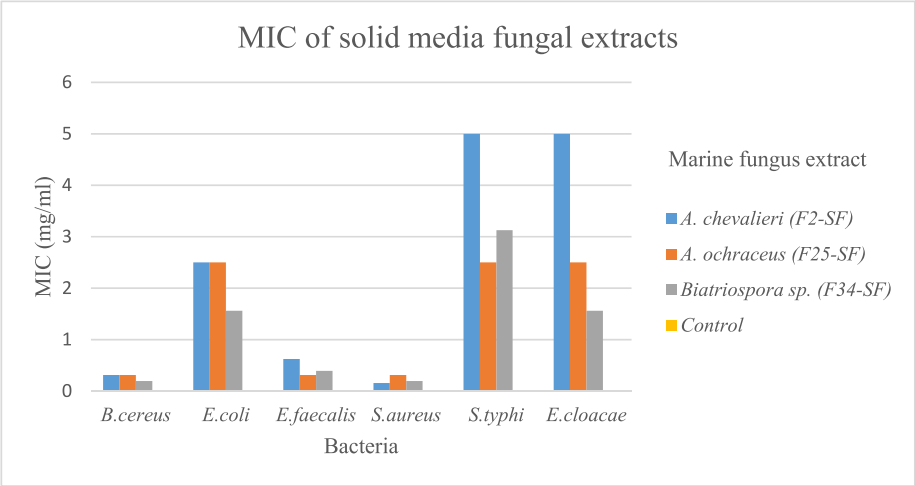

Fig. 7. MIC of solid media fungal extracts against six bacteria.

Table 2
Antioxidant potential and total phenolic content of the fungal extracts.

Extract	DPPH scavenging activity (IC_{50} value in μg/ml)	FRAP (mM Fe^{2+}/g DWE)	Total phenolic content (mg GAE/g DWE)
A. chevalieri (F2M)	88.40 ± 5.51 ^{de}	3.593 ± 0.060 ^{c}	173.14 ± 4.47 ^{b}
A. ochraceus (F25M)	687.47 ± 20.45 ^{a}	1.035 ± 0.013 ^{e}	32.05 ± 0.16 ^{d}
*Biatriospora*sp. (F34M)	314.43 ± 4.10 ^{c}	5.02 ± 0.057 ^{b}	62.14 ± 0.68 ^{c}
A. chevalieri (F2-SF)	78.92 ± 4.71 ^{e}	11.17 ± 0.20 ^{a}	360.35 ± 10.31 ^{a}
A. ochraceus(F25-SF)	589.72 ± 16.40 ^{b}	0.896 ± 0.025 ^{e}	41.16 ± 0.43 ^{d}
Biatriospora sp. (F34-SF)	115.24 ± 3.95 ^{d}	2.16 ± 0.048 ^{d}	178.22 ± 1.43 ^{b}
Positive control	12.19 ± 0.82	–	–

Statistical analyses were performed using one-way ANOVA followed by Tukey post hoc. Different superscripts between rows in individual columns represent significant differences between extracts.

Table 3
Enzymatic index of the three isolates.

Enzyme Assay	F2	F25	F34
Amylase	1.05	1.17	1.92
DNAse	2.78	1.09	1.03
Lipase	2.60	1.11	–
Protease	–	1.16	1.12
Cellulase	–	1.11	–
Gelatinase	–	+	+
Urease	–	+	+

β-tubulin (TUB2) and the RNA polymerase II second largest subunit (RPB2) are normally included in the taxonomy [18,42].

The *Aspergillus* genus is ubiquitous and many species have been isolated as endophytes, saprophytes and even pathogens in the marine environment. Phylogenetic analyses using the concatenated datasets herein have allowed the correct identification of isolates *Aspergillus chevalieri* (F2) and *Aspergillus iizukae* (F37). In the previous study by Wong Chin et al. [34], these isolates were believed to be *Aspergillus cristatus* and *Aspergillus flavipes*, respectively. The maximum likelihood tree obtained by Siqueira et al. [43] showed that *A. chevalieri* and *A. cristatus* are closely related. According to Hubka et al. [44], *A. iizukae* and *A. flavipes* also belong to the same clade; the *Flavipedes* clade. Therefore, for proper species level identification, ITS sequence data has to be analysed together with protein-coding genes, in order to distinguish between closely related strains. The multigene phylogeny provided further evidence to support that isolate F25 was *Aspergillus ochraceus*. *Aspergillus ochraceus* has been reported both in the terrestrial and marine environment. It is also a known food pathogen which produces the carcinogenic mycotoxin ochratoxin A, penicillic acid, dihydropenicillic acid and viomellein. Frank et al. [45] have isolated this species from the inner tissue of Mediterranean sponge *Agelas oroides*, indicating that it exists a sponge endosymbiont.

The genus *Exserohilum*, also known as *Setosphaeria*, contains plant, human and animal pathogens, saprophytes and endophytes. These can thrive in different environments ranging from arid soils to the oceans [46–48]. It was confirmed that isolate F33 was

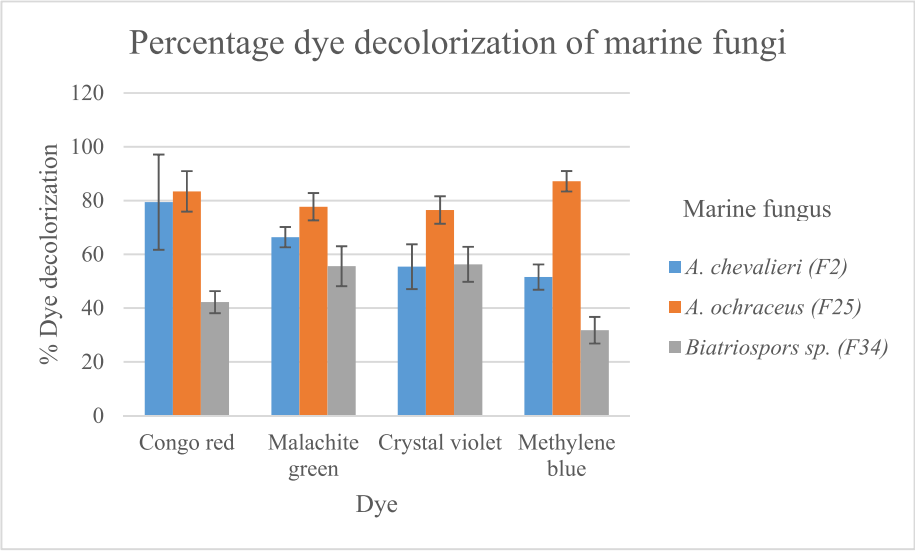

Fig. 8. Percentage dye decolorization of the three isolates.

Exserohilum rostratum, based on the multigene phylogenetic analyses. Pang et al. [47] also isolated a sponge-derived *Setosphaeria* sp. SCSIO41009, near Xuwen County, Guandong Province, China. It is not unusual to isolate this sponge associated strain from the marine environment.

Biatriospora is rather unexplored and has often been mistaken for *Nigrograna*. Isolates, belonging to this genus, are mainly causal agents of infections, decomposers and endophytes [49,50]. Species level identification was not possible for isolate F34, due to the inadequacy of TUB2 and ACT sequences available on this genus. Nevertheless, the ITS and LSU concatenated sequence dataset showed that isolate F34 was closely related to *Biatriospora antibiotica* CCF 4998, leading to the conclusion that F34 was *Biatriospora* sp.

Media conditions have an effect on the production of fungal secondary metabolites [36,51,52]. There are two types of fungal culture; solid-state fermentation or submerged fermentation. The use of a solid substrate, such as rice, oatmeal or wheat, provides an environment with low water concentration which facilitates oxygen transfer during growth of the fungi. This often results in higher concentrations of metabolites being produced. Submerged fermentation, using nutrient media like potato dextrose broth, Czapek Dox, yeast extract peptone dextrose and malt extract broth, is preferred when culture parameters have to be monitored closely [36,53]. In this study, the two most commonly used media, potato dextrose broth and rice, were employed for secondary metabolite production.

Marine fungi produce a plethora of bioactive secondary metabolites that have antimicrobial properties. *Aspergillus chevalieri* (F2) and *A. ochraceus* (F25) have shown good antimicrobial properties against the bacteria used in this study. About 361 natural bioactive compounds have been reported in marine *Aspergillus* species [54]. The compounds cryptoechinulin A, neoechinulin B, 7-prenyleoechinulin B, neoechinulin D, variecolorin H were isolated from a marine *A. chevalieri* MCCC M23426. These compounds, obtained from solid fermentation on rice/millet media, showed potent antimicrobial activity against *Staphylococcus aureus* [55]. LC-MS studies by Attia et al. [56] have shown that *A. ochraceus* produces the antimicrobial compounds versicolin, terreic acid, fumigatin, aspyrone, 4-hydroxymellin and terremutin when grown in PDB medium. These compounds might also be produced by the isolates in this study, accounting for their antimicrobial activity. The genus *Biatriospora* produces diverse secondary metabolites which can be potential antibiotics [57,58]. The *Biatriospora* sp. CIGOM2 produced the metabolites cytochalasin D, benzylisoquinoline alkaloid and meleagrin A. This deep-sea isolate, when grown in rice media with Czapek-Dox or artificial marine water, showed good antimicrobial properties in the study by Villanueva-Silva et al. [59]. There was no significant difference between the antimicrobial activity of the liquid fermentation and the solid media fermentation extracts. Therefore, the antimicrobial compounds were produced by the fungi when grown in both media. Li et al. [35] also used PDB medium and rice medium for secondary metabolite extraction of *Aspergillus* sp. LS34. They obtained five compounds from rice medium and four compounds from PDB medium. Most of these compounds showed antimicrobial properties against the pathogenic bacteria *Vibrio parahaemolyticus*, *V. harveyi*, *Escherichia coli* and *Staphylococcus aureus*.

An antioxidant has the capacity to reduce the concentration of ROS, which otherwise could cause cell damage and disease. The solid media extract of *Aspergillus chevalieri* (F2-SF), showed higher antioxidant potential, as compared to the two other isolates. The seaweed endophyte *A. chevalieri*, obtained by Calado et al. [52,60], also showed the highest antioxidant ability among all the tested extracts. It showed EC_{50} of 35.5 μg/ml in the DPPH scavenging assay, TPC 402.9 ± 42.0 mg GA/g and FRAP 3256.1 ± 174.3 μM $FeSO_4$/g. The chemical analyses showed the presence of the two bioactive compounds, echinulin and neoechinulin. There was a significant difference between the IC_{50} of the mycelium liquid media extracts and the solid media extracts. According to VanderMolen et al. [36], the production of marker compounds was detected in both liquid and solid media. Nevertheless, greater quantities of the marker compounds were produced in the rice media. Hence, the isolates might produce greater quantities of antioxidant compounds when cultivated in the rice media.

Fungi are known to produce extracellular enzymes like phosphatase, lipase, cellulase, amylase, laccase and proteases. These

enzymes have important biotechnological applications in the food and beverages, textile, detergent, paper and pulp, pharmaceutical and medical industries [61]. *Aspergillus ochraceus* produces a variety of enzymes which have biotechnological and industrial values as reported by Hareeri et al. [62]. Isolate F25 was no different and produced the seven enzymes included in our study. Although being a sponge-associated fungus, it was equipped with cellulose degrading enzymes. *Biatriospora carollii* CCF 4484, isolated from sediments by Orwa et al. [63], did not produce amylase or protease. Isolate F34, from this study, was able to degrade these substrates. According to Balabanova et al. [29], marine fungi are able to specialize to the substrates they encounter frequently in the marine environment. This might account for the differences observed with our isolates.

The removal of dye colour by marine fungi can be applied in bioremediation of wastewater treatment plants and in coastal waters. This decolorization potential is attributed to the secretion of extracellular enzymes and/or biosorption by the fungal biomass [64]. The genus *Aspergillus* has been studied for its dye decolorization properties. Assess et al. [65] have shown that *A. niger* was able to decolorize 97% of 200 mg/l of Congo red during 6 days, at pH 5 under shaking conditions. This property was attributed to the lignin peroxidase and manganese peroxidase production. Since isolate F25 was able to produce various enzymes, its dye decolorization potential might be attributed to extracellular enzyme production. The three fungi produced different combinations of enzymes and showed specificity in dye decolorization. This might indicate different mechanisms of action.

5. Conclusion

This is the first study to employ multigene phylogenetic analyses to ascertain the identity of marine fungi associated with sponges and brown algae in Mauritius. Furthermore, there are no reports on the antimicrobial and antioxidant properties of solid media extracts, enzyme production and dye decolorization potentials of marine fungi living in the Mauritian waters. Using multigene phylogenetic analyses, *A. chevalieri* and *A. iizukae* were identified from brown algae while *A. ochraceus*, *E. rostratum* and *Biatriospora* sp. were obtained from sponges. The *Aspergillus chevalieri* strain possessed good antimicrobial properties and had lowest MIC against the human pathogen *S. aureus*. Moreover, it had high antioxidant potential in the assays that were performed. The fungi also produced extracellular enzymes that help them in dye decolorization. *A. ochraceus* was able to produce all the enzymes under study and had the highest dye decolorization potential against Methylene blue. These interesting properties have to be further studied as marine fungi have the potential to be useful in the industry. The chemical compounds responsible for the strong antimicrobial and antioxidant properties have to be determined and purified. Moreover, the different mechanisms of action of the marine fungi in the dye decolorization process have to be investigated in order to exploit them further.

Ethics approval

This article does not contain any studies with humans or animals.

Data availability statement

The sequences obtained in this study were deposited in GenBank under accession number OQ754174 – OQ941652.

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CRediT authorship contribution statement

Jessica Mélanie Wong Chin: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization, Methodology. **Daneshwar Puchooa:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Theeshan Bahorun:** Writing – review & editing, Supervision. **Abdulwahed Fahad Alrefaei:** Writing – review & editing, Funding acquisition. **Vidushi S. Neergheen:** Writing – review & editing, Validation, Supervision, Formal analysis. **Rajesh Jeewon:** Resources, Project administration, Funding acquisition, Conceptualization, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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