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Diversity and antimicrobial activity of culturable fungi associated with sea anemone *Anthopleura xanthogrammica*

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ABSTRACT

Background: The main objective of this study was to isolate fungi associated with *Anthopleura xanthogrammica* and measure their antimicrobial and enzymatic activities. A total of 93 fungal strains associated with *A. xanthogrammica* were isolated in this study, of which 32 isolates were identified using both morphological characteristics and internal transcribed spacer (ITS) sequence analysis. The antibacterial activities of 32 fungal isolates were tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Edwardsiella tarda*, *Vibrio harveyi*, *Fusarium oxysporum*, and *Pyricularia oryzae* by agar diffusion assay. Extracellular hydrolytic enzyme activities of the fungal isolates were determined by agar diffusion assays. Enzyme activities were detected from clear halo size.

Results: The isolated fungi belonged to 18 genera within 7 taxonomic orders of 1 phylum. The genera *Aspergillaceae* were the most diverse and common. The antimicrobial activities of 32 isolates were evaluated, and 19 (59.4%) of fungi isolate displayed unique antimicrobial activities. All fungal strains displayed at least one enzyme activity. The most common enzyme activities in the fungi isolates were amylase and protease, while the least common were pectinase and xylanase.

Conclusions: This is first report on the sea anemone-derived fungi with antimicrobial and enzyme activities. Results indicated that sea anemone is a hot spot of fungal diversity and a rich resource of bioactive natural products.

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1. Introduction

Antibiotics have been playing an important role in fighting against infectious diseases caused by microbes. Excessive use of antibiotics has developed health problem due to diseases caused by drug-resistant pathogens [1]. Consequently, there is a growing urgent demand for new antibacterial and antifungal compounds [2]. The frequency of discovery of new antimicrobial compounds has dropped [3]. There is a need to find new antimicrobial compounds.

Antimicrobial proteins (AMPs) are widely distributed in nature [4]. Filamentous fungi are well known for the production of antimicrobial agents, industrial enzymes, and microbial biomass [5]. A number of fungal species are well known for the production of industrial enzymes [6,7]. A significant number of secondary metabolites have been reported from fungal strains associated with the invertebrates [8].

Marine microorganisms have been found to be the most versatile source for novel antibacterial and antifungal compounds [9]. Among all the marine microorganisms, marine fungi are considered as a prolific resource of antimicrobials discovery because they show a high biodiversity and produce a wide variety of biologically active secondary metabolites [2]. Marine fungi associated with invertebrates have received tremendous attention [10].

Phylum Cnidaria includes nearly 10,000 species and of which corals, jellyfishes and sea anemones are predominant. Among these, the sea anemones have more potential to produce bioactive compounds and are promising sources of bioactive compounds of medical interest [11]. In common with sponges and corals, sea anemones produce many biologically active polypeptides and proteins [12]. They have

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evolved the ability to synthesize novel metabolites including natural products and enzymes, which could be used for novel antimicrobial drugs, many of which exhibit novel structural features, not found in terrestrial natural products. Fungi associated with a sea anemone *A. xanthogrammica* have not been studied previously; therefore, we have chosen to study antimicrobial and enzymatic activities of fungi associated with a sea anemone *A. xanthogrammica* in this study.

In the present study, sea anemone *A. xanthogrammica*-derived fungal strains were identified and their antimicrobial and extracellular enzymatic activities were assayed. This is the first study on the fungal endophytes associated with *A. xanthogrammica*. This study will enhance our knowledge regarding the diversity of fungal strains associated with *A. xanthogrammica*.

2. Material and methods

2.1. Sample sites and sample collection

Healthy *A. xanthogrammica* samples were collected from 10 stations between 119°27′83″ to 119°28′98″ E and 34°46′5″ N in Haizhou bay, China. The samples were transferred directly to sterile plastic zip-lock bags and stored in an icebox.

2.2. Fungi isolation

The samples were rinsed three times with sterile seawater to remove sediments and loosely attached microorganisms [13]. The surface of the samples were sterilized with 75% ethanol for 30 s and washed three times with sterile seawater. The resulting homogenate was further diluted 10 times with sterile seawater and 100 μL dilution was plated onto the two agar plates of the following media: potato dextrose agar (PDA) medium (PDA, Difco™) and Czapek-Dox agar (CDA) medium (Sigma Aldrich).

2.3. DNA extraction, PCR, and identification

Genomic DNA was extracted from fungal cultures using the Fungal DNA (*E.Z.N.A.*®, Omega). The resulting genomic DNA was used as a template to amplify fungal ITS-rDNA fragments using the primers ITS1 (TCCGTAGGTGAACCTGCG) and ITS4 (TCCTCCGCTTATTGATATGC) [14]. The PCR reaction was performed in 50 μL of reaction mixture

Table 1
Phylogenetic affiliations of fungi associated with *A. xanthogrammica*.

NO.	Isolate ID (accession no.)	Order	Genus	Closest identified relative	Query cover (%)	Identity (%)
1	CAF093 (KU821452)	Capnodiales	*Cladosporium*	*Cladosporium tenuissimum* (AJ300331)	99	97
2	CAF059 (KU821453)			*Cladosporium allicinum* (KP701975)	99	99
3	CAF032 (KU821454)			*Cladosporium cladosporioides* (KP942857)	99	99
4	CAF011 (KY420933)			*Cladosporium sphaerospermum* (KT962859)	100	99
5	CAF001(KY420934)			*Cladosporium halotolerans* (KP701911)	100	99
6	CAF111(KY420935)			*Cladosporium colocasiae* (KY310656)	98	99
7	CAF025 (KU821455)		*Neodevriesia*	*Teratosphaeria capensis* (JN712501)	97	95
8	CAF005 (KU821456)	Dothideales	*Diaporthe*	*Diaporthe leucospermi* (KT323121)	99	99
9	CAF036 (KU821457)			*Diaporthe arecae* (KT207761)	96	91
10	CAF004 (KY420945)			*Diaporthe phoenicicola* (KT207761)	100	100
11	CAF027 (KU821458)			*Diaporthe macintoshii* (KJ197289)	99	97
12	CAF068 (KU821459)			*Diaporthe lithocarpus* (KR703276)	97	99
13	CAF019 (KU821460)	Eurotiales	*Penicillium*	*Penicillium chrysogenum* (AY373902)	100	99
14	CAF079 (KU821462)			*Penicillium fellutanum* (JQ724525)	100	99
15	CAF003 (KU821463)			*Penicillium adametzioides* (KF143792)	100	99
16	CAF052 (KU821464)		*Aspergillus*	*Aspergillus ochraceus* (FJ878632)	97	97
17	CAF066 (KU821467)			*Aspergillus ostianus* (FJ478090)	96	94
18	CAF012 (KU821468)			*Aspergillus ochraceopetaliformis* (FJ797698)	95	97
19	CAF071 (KU821471)			*Aspergillus homomorphus* (NR077189)	98	92
20	CAF053 (KU821472)			*Aspergillus insulicola* (FR733834)	97	99
21	CAF085 (KU821473)	Sordariales	*Sordariaceae*	*Neurospora tetrasperma* (JX136749)	99	99
22	CAF090 (KU821474)			*Neurospora sitophila* (KM588213)	100	98
23	CAF034 (KU821475)		*Chaetomium*	*Chaetomium* sp. (EU035802)	97	99
24	CAF056 (KU821476)	Trichosphaeriales	*Nigrospora*	*Nigrospora oryzae* (EU272503)	99	93
25	CAF037 (KU821477)			*Nigrospora sphaerica* (HQ608063)	100	99
26	CAF046 (KU821478)			*Nigrospora* sp. (GU017506)	99	99
27	CAF062 (KU821479)	Pleosporrales	*Microsphaeropsis*	*Microsphaeropsis arundinis* (JX496010)	99	99
28	CAF087 (KU821480)			*Microsphaeropsis* sp. (HQ914808)	98	99
29	CAF006 (KU821481)		*Massarina*	*Massarina eburnean* (AF383959)	96	99
30	CAF081 (KU821482)	Hypocreales	*Trichoderma*	*Trichoderma harzianum* (KR856225)	97	99
31	CAF023 (KU821483)			*Trichoderma guizhouense* (KP115286)	100	99
32	CAF060 (KY420936)		*Myrothecium*	*Myrothecium masonii*	100	100

containing 25 μL of 2 × Taq PCR Master mix (Takara), 2 μL of forward primer (10 mM), 2 μL of reverse primer (10 mM), 2 μL of template DNA and 19 mL of sterile double-distilled water. The PCR products were sequenced with the primer ITS4 or ITS1. The sequences were then analyzed with Nucleotide-Nucleotide BLAST (BLASTn).

2.4. Phylogenetic analysis

Lasergene Software SeqMan (DNAStar Inc.) was used to edit fungal ITS-rDNA sequences. Each of these sequences were compared against all DNA sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST). All fungal ITS sequences were aligned using the Clustal X (1.83) software. Neighbor-joining (NJ) trees were generated using MEGA 4.0 combined with bootstrap analysis with 100 replicates. Fungal ITS sequences of the representative isolates were deposited in GenBank under accession numbers KU821452–KU821483 (Table 1).

2.5. Antimicrobial activity of fungi

The extracts of fungi were prepared with the method described previously [15]. The antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Edwardsiella tarda, Vibrio harveyi*, *Fusarium oxysporum*, and *Pyricularia oryzae were* determined in triplicate by agar diffusion assay, and the results were expressed as mean ± standard error of mean (SEM).

2.6. Extracellular hydrolytic enzyme activity assay

All agar diffusion assays for the detection of enzyme activities were performed on solid PDA medium supplemented with appropriate dissolved substrate for screening extracellular enzymes. The dissolved substrates for screening amylase, cellulase, chitinase, lipase, pectinase, xylanase, and protease were 0.2% soluble starch, 0.5% carboxymethylcellulose, 2.5% purified chitin, 1% tributyrin, 1% pectin, 0.5% xylan, 2% casein, respectively. The plates were incubated at 30°C for 3–6 d, and enzyme activities were detected from clear halo size. All the enzyme assays were carried out in triplicate, and the results were expressed as mean ± standard error of mean (SEM).

3. Results and discussion

3.1. Isolation and analysis of culturable fungi associated with *A. xanthogrammica*

To evaluate the diversity of fungi associated with *A. xanthogrammica,* a total of 93 fungal strains were isolated. After excluding duplicate strains based on morphological and taxonomic characteristics, 32 fungal isolates were selected. A neighbor-joining phylogenetic tree constructed using rDNA-ITS sequences of cultured fungi from *A. xanthogrammica* in Haizhou Bay is shown in Fig. 1. The identified fungi and their best matches are summarized in Table 1.

Thirty-two fungal isolates were selected based on the BLAST and phylogenetic analysis using the rDNA-ITS sequences. All fungal isolates belong to the phylum Ascomycota including 7 taxonomic orders: Capnodiales, Dothideales, Eurotiales, Sordariales, Trichosphaeriales, Pleosporrales, Hypocreales, and Xylariales. Most of the isolates matched their closest relatives with 97% to 100% similarity except for CAF036 (91%), CAF071 (92%), CAF056 (93%), and CAF025 (95%) (Table 1).

Phylogenetic analysis showed that the isolated fungi belonged to Ascomycota embracing 11 genera (*Cladosporium*, *Neodevriesia*, *Diaporthe*, *Penicillium*, *Aspergillaceae*, *Sordariaceae*, *Chaetomium*, *Nigrospora*, *Microsphaeropsis*, *Massarina* and *Trichoderma)* in seven orders. Eurotiales was the dominant group of the identified fungi accounting for 36.7%, among which *Aspergillus* was predominant with 9/32 isolates.

Considerable attention has been given to isolate the fungal strains associated with marine invertebrates such as sponges and corals, but much less is known about the fungal strains associated with sea anemone. The isolated fungi strains in this study belonged to Ascomycota. Based on our current knowledge, no similar studies have been reported to date. Some studies have explored the effects of

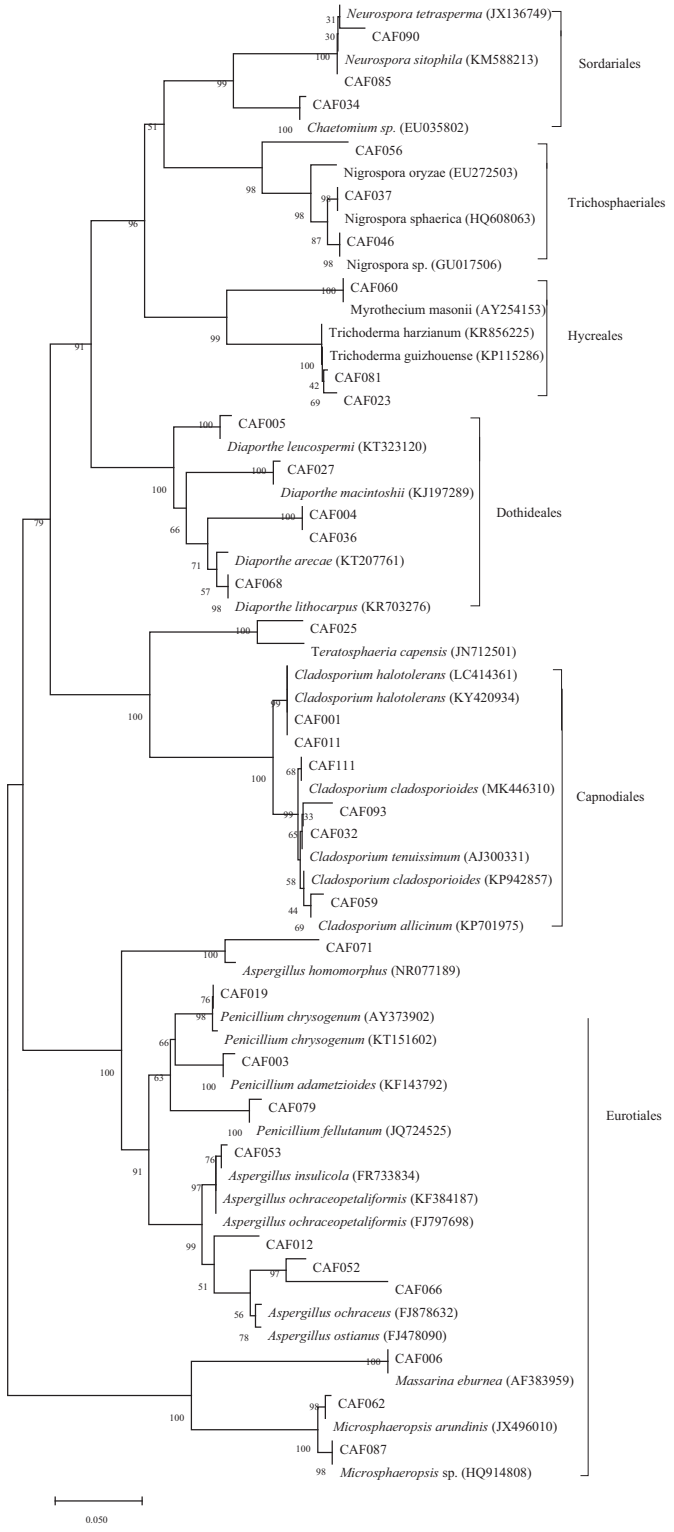

Fig. 1. Neighbor-joining unrooted phylogenetic tree constructed using rDNA-ITS sequences of cultured fungi from *Anthopleura xanthogrammica* in Haizhou Bay. Number at branch indicates Bootstrap value (>50%) of neighbor-joining analysis from 100 replicates.

environmental conditions or the physiological state of the host on fungal communities. The nature of these interactions remains unclear. It is well known that sampling place affects the recovered strain diversity. In future studies, different other sites will be explored to access fungal diversity associated with sea anemone.

3.2. Screening for antimicrobial activity

A total of 32 isolates were tested against *B. subtilis*, *S. aureus*, *E. coli*, *E. tarda*, *V. harveyi*, *F. oxysporum*, and *P. oryzae* to detect their antimicrobial activities. As showed in Tables 2, 19 strains (59.4%) of fungi showed different antimicrobial activities against the tested strains. Overall, the isolates showed higher percentage of antibacterial activity than that of antifungal activity. Fungal isolates were active against the gram-positive *S. aureus* (43.7%), *B. subtilis* (50.0%), *E. coli* (43.7%), *E. tarda* (34.3%) and *V. harveyi* (37.5%). However, for the antifungal activity, the percentage of antibacterial activity is less than 21.8%, especially for the antimicrobial activity for *S. cerevisiae*, the percentage was only 12.5%.

Different fungal strains exhibited different antimicrobial activities. Capnodiales and Pleosporrales orders fungal strains showed highest antifungal activity against two pathogenic filamentous fungi *F. oxysporum* and *Pyricularia grisea*. Additionally, three fungal isolates, *Aspergillus ochraceopetaliformis* CAF012, *Cladosporium cladosporioides* CAF032, and *Aspergillus homomorphus* CAF071 displayed antimicrobial activity toward all the tested microorganisms. *Aspergillus ochraceus* CAF052 strongly inhibited all tested bacteria including two aquatic pathogenic bacteria *E. tarda* and *V. harveyi*. Furthermore, *Aspergillus ostianus* CAF066 and *A. homomorphus* CAF071 strongly inhibited two plant pathogenic fungi *F. oxysporum* and *P. grisea*.

Overall, the isolates showed higher percentage of antimicrobial activity than that of antifungal activity. In this study, 59.4% of fungi demonstrated antimicrobial activity, which is in accordance with previous reports that showed 20–70% of culture microorganisms showed antimicrobial activity [16,17]. These strains could produce interesting and useful antimicrobial compounds with the potential to serve as drugs or drug leads.

Aspergillus is well known for the production of bioactive natural products with antimicrobial activities [18]. Several interesting antibacterial agents have been reported from *Penicillium* [19]. Endophytic fungi isolated from *Eugenia jambolana* have shown effective antibacterial activity against multidrug-resistant organisms [20].

Microbial strains isolated from sea anemone *Anemonia sulcata* and *Actinia equine* showed antimicrobial activities [21].

The antibacterial and antifungal activities found in this study are very promising, and these fungal strains have potential for industrial-scale commercial production.

3.3. Extracellular hydrolytic enzyme activity assay

The agar diffusion method was used to determine enzyme activities from 32 fungal strains. As shown in Table 3, all strains displayed at least one enzyme activity. The most common enzyme activities in the fungi isolates were amylase and protease, while the least common were pectinase and xylanase (Table 3). Most of the isolates exhibited 2 to 5 enzyme activities. Four isolates, namely *Penicillium chrysogenum* CAF019, *Trichoderma harzianum* CAF081 and *Trichoderma guizhouense* CAF023, exhibited 7 enzyme activities. *Microsphaeropsis arundinis* CAF062 only showed protease activity and *Diaporthe arecae* CAF036 only exhibited amylase activity.

Fungi are well known for the production of enzymes. Because fungi excrete enzymes into the media and enzyme levels are also much greater than those of excreted by yeast and bacteria, fungi are considered to be particularly interesting producers of enzymes [22,23].

Fungi are well known for the production of industrial enzyme [6]. Different fungal strains isolated in this study have shown amylase, cellulase, chitinase, lipase, pectinase and xylanase activities. Amylase, cellulase, chitinase, lipase, pectinase and xylanase have important industrial applications [5]. Marine-derived fungi are well known for novel enzymes production and their enzymes are different than terrestrial fungal enzymes [24,25]. Fungal strains isolated in this study have the capability for industrial-scale enzyme production.

4. Conclusion

This study will help to further explore *A. xanthogrammica*–fungal association, which will lead to the industrial development of enzymes and antimicrobial agents. This is the first study of diverse fungal endophytes isolated from *A. xanthogrammica*. These results

Table 2
Antibacterial activity of the EtOAc extracts from the fermentation broth and mycelia of the active fungi.

Isolates	Species	*B. subtilis*	*S. aureus*	*E. coli*	*S. cerevisiae*	*E. tarda*	*V. harveyi*	*F. oxysporum*	*P. oryzae*
CAF093	*Cladosporium tenuissimum*	+	+	+	+	+	+++	−	−
CAF059	*Cladosporium allicinum*	+	+	+	−	+	+	−	−
CAF032	*Cladosporium cladosporioides*	+++	+	+++	+	+	+	+++	+
CAF011	*Cladosporium sphaerospermum*	+	++	++	+	+	+	+++	+++
CAF001	*Cladosporium halotolerans*	+++	+	+	+	+	+	+	+++
CAF111	*Cladosporium colocasiae*	+	+	+++	+	−	+	+	+
CAF036	*Diaporthe arecae*	−	+	−	−	−	−	−	−
CAF004	*Diaporthe phoenicicola*	−	−	+	+	−	+	−	−
CAF079	*Penicillium fellutanum*	−	−	−	−	+++	+	−	−
CAF003	*Penicillium adametzioides*	+	+	+++	−	+++	+++	+	+
CAF052	*Aspergillus ochraceus*	+++	+++	+++	−	+++	+++	−	−
CAF066	*Aspergillus ostianus*	−	−	+++	−	+	−	+++	+++
CAF012	*Aspergillus ochraceopetaliformis*	+	+	+++	+++	+	+	+	+
CAF071	*Aspergillus homomorphus*	+	+++	+	+	+	+++	+++	+++
CAF056	*Nigrospora oryzae*	+	+	+	−	+	+	+	+
CAF037	*Nigrospora sphaerica*	+	+	−	−	−	−	−	−
CAF006	*Massarina eburnean*	+	−	+	−	−	+	−	−
CAF081	*Trichoderma harzianum*	+	+	−	−	−	−	−	−
CAF023	*Trichoderma guizhouense*	−	+	−	−	−	−	−	−

Negative control: dimethyl sulfoxide (DMSO).
−: no antibacterial activity.
+: diameter of zone of inhibition less than 10 mm.
++: diameter of zone of inhibition is from 10 mm to 15 mm.
+++: diameter of zone of inhibition larger than 15 mm.

Table 3
List of fungi screened for enzymes on solid media.

Isolate ID	Closest identified relative	Enzyme activities halo (mm[a])						
		Amylase	Cellulase	Chitinase	Lipase	Pectinase	Xylanase	Protease
CAF093	*Cladosporium tenuissimum*	6.36 ± 0.35	8.59 ± 0.17	–	3.91 ± 0.31	4.83 ± 0.31	–	–
CAF059	*Cladosporium allicinum*	7.12 ± 0.23	4.32 ± 0.36	–	–	–	–	3.12 ± 0.22
CAF032	*Cladosporium cladosporioides*	–	6.37 ± 0.33	–	8.98 ± 0.85	6.85 ± 0.26	13.39 ± 1.02	7.65 ± 0.57
CAF011	*Cladosporium sphaerospermum*	6.51 ± 0.27	3.11 ± 0.26	–	–	–	–	3.05 ± 0.25
CAF001	*Cladosporium halotolerans*	4.41 ± 0.25	–	–	–	–	–	3.09 ± 0.27
CAF111	*Cladosporium colocasiae*	3.57 ± 0.13	2.87 ± 0.23	–	–	–	–	3.13 ± 0.26
CAF025	*Teratosphaeria capensis*	–	–	–	–	–	–	2.67 ± 0.75
CAF005	*Diaporthe leucospermi*	–	–	–	2.12 ± 0.37	–	–	2.31 ± 0.56
CAF036	*Diaporthe arecae*	4.26 ± 0.26	–	–	–	–	–	–
CAF004	*Diaporthe phoenicicola*	3.65 ± 0.35	–	–	–	–	–	–
CAF027	*Diaporthe macintoshii*	–	3.56 ± 0.35	3.95 ± 0.32	–	–	–	–
CAF068	*Diaporthe lithocarpus*	4.52 ± 0.18	–	–	–	–	–	4.77 ± 0.21
CAF019	*Penicillium chrysogenum*	5.97 ± 0.31	3.54 ± 0.26	3.18 ± 0.61	3.62 ± 0.51	5.36 ± 0.31	5.89 ± 0.18	8.65 ± 0.46
CAF079	*Penicillium fellutanum*	7.25 ± 0.65	5.93 ± 0.23	–	3.87 ± 0.32	3.21 ± 0.27	–	5.63 ± 0.27
CAF003	*Penicillium adametzioides*	–	12.27 ± 0.32	3.91 ± 0.34	5.29 ± 0.21	–	–	–
CAF052	*Aspergillus ochraceus*	8.78 ± 0.69	3.26 ± 0.19	–	–	–	7.95 ± 0.41	5.67 ± 0.38
CAF066	*Aspergillus ostianus*	5.18 ± 0.11	4.19 ± 0.31	–	–	3.99 ± 0.38	–	–
CAF012	*Aspergillus ochraceopetaliformis*	5.32 ± 0.27	–	–	3.98 ± 0.26	–	–	–
CAF071	*Aspergillus homomorphus*	11.76 ± 0.39	–	3.76 ± 0.33	–	–	–	8.19 ± 0.32
CAF053	*Aspergillus insulicola*	12.58 ± 0.12	–	–	6.98 ± 0.33	–	–	2.15 ± 0.36
CAF085	*Neurospora tetrasperma*	–	3.76 ± 0.21	7.83 ± 0.74	–	–	–	–
CAF090	*Neurospora sitophila*	–	2.32 ± 0.18	2.76 ± 0.55	–	–	–	–
CAF034	*Chaetomium* sp.	3.53 ± 0.31	–	5.98 ± 0.35	7.85 ± 0.32	3.52 ± 0.32	2.38 ± 0.34	6.37 ± 0.31
CAF056	*Nigrospora oryzae*	3.11 ± 0.35	–	–	4.65 ± 0.28	–	–	3.23 ± 0.22
CAF037	*Nigrospora sphaerica*	–	4.53 ± 0.21	–	5.44 ± 0.62	–	–	5.27 ± 0.75
CAF046	*Nigrospora* sp.	3.32 ± 0.12	3.23 ± 0.32	5.63 ± 0.22	9.23 ± 0.36	–	–	–
CAF062	*Microsphaeropsis arundinis*	–	–	–	–	–	–	6.23 ± 0.65
CAF087	*Microsphaeropsis* sp.	4.12 ± 0.37	–	–	3.96 ± 0.31	–	–	4.32 ± 0.13
CAF006	*Massarina eburnean*	12.31 ± 0.32	–	2.53 ± 0.26	–	–	–	–
CAF081	*Trichoderma harzianum*	9.53 ± 0.28	5.61 ± 0.41	4.39 ± 0.31	8.62 ± 0.73	6.54 ± 0.37	3.65 ± 0.62	3.23 ± 0.27
CAF023	*Trichoderma guizhouense*	3.67 ± 0.38	3.98 ± 0.32	5.23 ± 0.27	5.23 ± 0.66	5.23 ± 0.23	3.29 ± 0.35	4.18 ± 0.31
CAF060	*Myrothecium masonii*	4.14 ± 0.32	5.07 ± 0.31	5.12 ± 0.21		–	–	9.67 ± 0.39

[a] Measured from the edge of the colony to limit of the halo.

suggest that fungal endophytes isolated from *A. xanthogrammica* are a prolific resource for the discovery of bioactive natural product. These results confirmed that fungal strains derived from *A. xanthogrammica* have antimicrobial and enzymatic activities. Our study contributes to *A. xanthogrammica*-associated fungi with novel antimicrobial and enzymatic activities.

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Conflict of interest

The authors declare that they have no conflict of interest.

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