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ISOLATION AND CHARACTERIZATION OF FUNGI ASSOCIATED WITH BODY AND EGG OF DISEASED AFRICAN CATFISH, *Clarias gariepinus*

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Abstract: African catfish *(Clarias gariepinus)* is in high demand in the Malaysian market. However, the species is known to be easily infected with diseases such as fungi, due to poor water quality and bad handling, which can cause mortality and economic losses. In addition, fungal infection in fish eggs can reduce their production. Four African catfish were purchased from local markets in Padang Hiliran, Kuala Terengganu and Kampung Manir, Kuala Terengganu, Terengganu, Malaysia. The objective of this study was to isolate and identify fungi from infected African catfish and their eggs. Affected fish with symptoms such as a swollen lesion, irregular swimming, and dark discolouration were screened for pathogenic fungi. Isolation, identification, and extracellular enzymatic assays were performed for the isolated fungi. Potato dextrose agar was used as an isolation medium for isolating fungi on African catfish and eggs. Fungi were identified by macroscopic observation of growth morphology and their structure, followed by microscopic observation after staining with lactophenol cotton blue to observe hyphae. Three fungi were isolated and identified as *Mucor* sp. (n=2) and *Trichoderma* sp. (n=1). All isolated fungi were also analyzed for extracellular enzymes, such as proteases, amylases, and lipases, to test their pathogenicity. *Trichoderma* sp. showed positive results in all tests. Moreover, a strain of *Mucor* sp. reacted positively to the amylase and lipase test while the other strain of the same species showed no reaction, indicating a negative result. In conclusion, isolated fungi have virulent properties and can cause mortality if the body and eggs are infected. Therefore, fish farms need comprehensive health management to prevent the spread of fungal diseases.

Keywords: African catfish, fungi infection, fish body, egg, extracellular enzymes.

Introduction

Aquaculture is important to support the growth of the world's population. Wild-caught or farmed fish are not sufficient to meet demand. The rapid growth in the production of carnivorous species, such as salmon, shrimp, and catfish, has been driven by the globalisation of trade, with intensive large-scale farming and advantageous economic conditions (Bostock *et al*., 2010). *Clarias gariepinus*, also known as African catfish, belongs to the Clariidae family. It is one of the most popular freshwater fish among farmers and consumers. It is in high demand due to its price, taste, relative resistance to poor water quality and ease of culture (Weyl *et al*., 2008).

In Malaysia, African catfish is a non-indigenous fish introduced through aquaculture from Thailand from 1986-1989 (Csavas, 1995). In the last two decades, this industry has developed tremendously and become the most productive fish cultured in both fresh and brackish water. It has also overtaken the production of red tilapia, which was previously the most farmed fish in Malaysia (Dauda *et al*., 2018). One of the most important problems in aquaculture is disease outbreaks caused by fungi, parasites, bacteria, and viruses, which can lead to economic losses; drugs and antibiotics cannot be avoided to treat the fish (Tartor *et al*., 2018; Ozkaya *et al*., 2017). Fungal diseases in fish and fish eggs are usually caused by the *Saprolegnia* species or oomycete

infections or "water mold" which are the second largest cause of economic losses in aquaculture (Van West, 2006).

The high losses in African catfish at the spawning stage are mostly due to fungal infections. However, few studies have been conducted on pathogenic fungi in fish farms and aquaculture (Melaku *et al*., 2017). Fungal infections can cause mortality rates of about 80-100%, which can lead to economic losses (Chukanhom & Hatai, 2004). Fungal infections are caused by poor water quality, improper sanitation and large amounts of decomposing organic material in the pond. It can cause the fish to exhibit lethargy, dark discolouration and loss of appetite (Leano, 2001; Patel *et al*., 2018). Fungi can become a problem when fish are stressed by disease, poor environmental conditions, poor nutrition, or injury. Fungi that infect fish eggs reduce production (Riede, 2004). The objective of this study was to isolate and identify fungi from infected African catfish and their eggs.

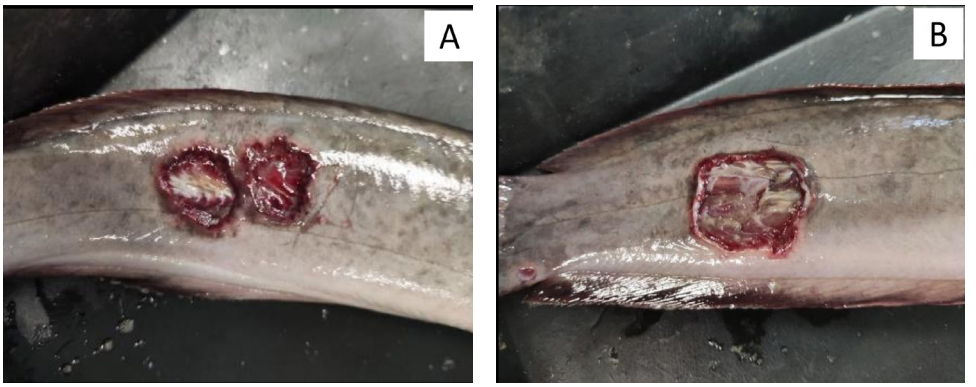
Materials and Methods

Sampling

Four African catfish were purchased from local markets in Padang Hiliran, Kuala Terengganu, Terengganu, Malaysia and Kampung Manir, Kuala Terengganu, Terengganu, Malaysia. Fish were selected based on the symptoms of ulcerative lesions. The fish were then stored in containers filled with fresh water and taken to the Aquatic Organism Health Laboratory at the Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, to isolate the fungi.

Fungi Isolation

Specimens were placed on a tray and clinical signs on the body were observed. The fish were then dissected and the eggs and gills were removed from the body. A sterilized cotton swab was used to isolate the fungi from the wound on the body and the eggs. Then, the cotton swabs were swabbed on potato dextrose agar (PDA) and incubated at 25^{o}C for about 3 to 4 days. After that, the fungi were transferred to new agar for pure culture. The pure culture was repeated continuously until only a single fungi species was present on the agar. Figures 1 shows symptoms of ulcerative lesions on the body. Figure 2 shows eggs from the African catfish.



Figures 1: Symptoms of ulcerative lesions on African catfish bodies (A) and (B) (arrow)

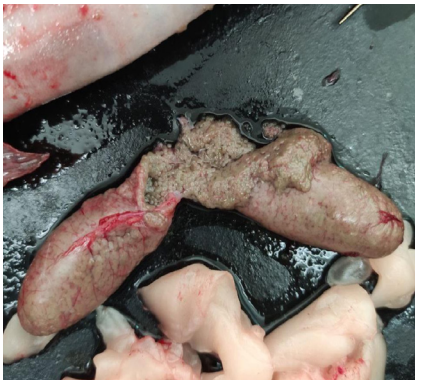


Figure 2: Eggs from the infected African catfish that shown symptoms of ulcerative lesions

Identification of Isolated Fungi Using Macro and Micro-Morphology

For micromorphology, the fungi were observed by sight and pictures were taken on agar. The fungi were observed by the shape and colour of the colonies. Three methods were used for micromorphology to observe isolated fungi under the microscope. In the first method, the fungus was transferred to the slide using a toothpick and a drop of lactophenol cotton blue. In the second method, a coverslip was pressed to the fungus and then placed on a slide containing lactophenol cotton blue. The last method entails pressing scotch tape on the fungus and then placing it on a slide containing lactophenol cotton blue. Another drop of lactophenol was added again and then covered with a coverslip. The slides were viewed under a microscope to look for structures such as hyphae, spores, and sporangia with conidiophores. Species were identified using an identification key according to Alsohaili and Bani-Hasan (2018).

Extracellular Enzyme Test

Virulence activities of isolated fungi were observed from the production of extracellular enzymes (proteolytic, lipolytic and amylolytic enzymes). All isolated fungi were cultured on agar for two to three days and incubated at 25°C in darkness. These methods adapted from Sanivada and Challa (2014), with some modifications, were used to detect haemolytic, proteolytic, amylolytic and lipolytic enzymes.

Proteolysis Test

The proteolysis assay was prepared using proteolysis agar. The agar mixture was prepared by mixing PDA powder with 1% skim milk powder and autoclaved at 120°C for 15 minutes. For the result, the proteolysis agar was flooded with an aqueous saturated solution of ammonium sulfate for about 30 minutes. A clear zone around the growth indicates the presence of the protease enzyme (positive result).

Amylase Test

The amylase test was prepared using a starch agar medium by mixing instant starch powder with distilled water and agar and autoclaving at 120°C for 15 minutes. The fungi cultured on starch agar were flooded with iodine solution for 30 minutes. A clear zone of hydrolysis around the growth was considered a positive result. The presence of a blue colour around the fungi indicated a negative result.

Lipase Test

Lipase medium agar was prepared by mixing 0.3% yeast extract, 0.5% pepsin and 0.1% granulated agar and autoclaved at 120°C for 15 minutes. The occurrence of clear medium was taken as a positive result.

Results

Isolation of Fungus from Diseased African Catfish

In this study, the method of morphological characterization was used to identify fungi isolated from African catfish. The distribution of the fungi isolated from the skin, eggs, and gills of the fish is presented in Table 1. The result showed that three fungi were isolated from the fish. The fungi were isolated from the wound on the body of fish 1 and 2. In fish 3, fungi were isolated from the eggs. However, no fungi were found in fish 4.

Table 1: Result for isolation of the fungus from diseased African catfish at different fish parts

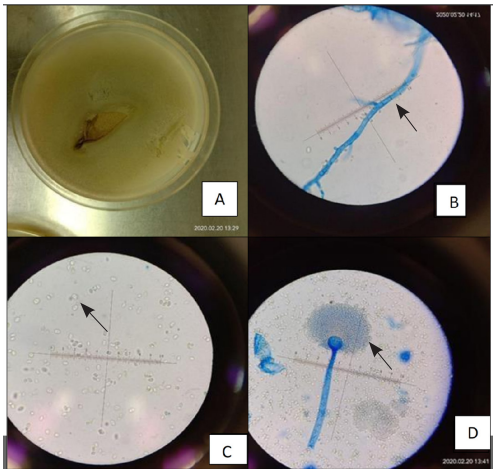
Fish	Locations/Parts		
	Body	Eggs	Gills
Fish 1	Present	Absent	Absent
Fish 2	Present	Absent	Absent
Fish 3	Absent	Present	Absent
Fish 4	Absent	Absent	Absent

Morphological Characterisation

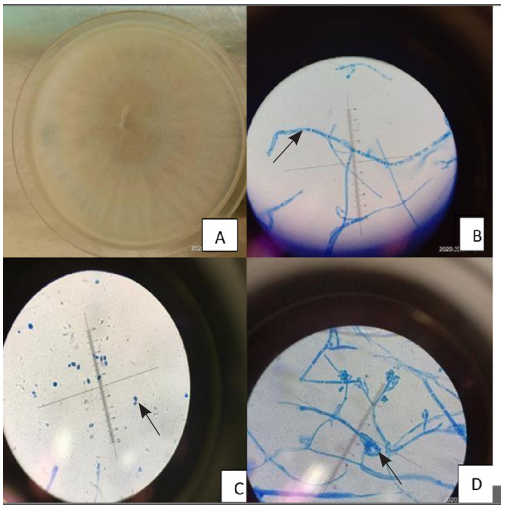
Three fungal isolates were obtained from infected African catfish. The morphological characteristics of the fungal isolates are shown in Table 2. Isolates 1 and 2 had the same morphological characteristics. The colour of the fungal growth was white to greenish and had coenocytic hyphae with globose sporangia and subglobose columella. Isolate 3 was a white colour with thick mycelium. It had septate hyphae, branched conidiophores, brush-like spores at the ends of conidiophores and oval conidia at the tip of phialides. Based on their morphological characteristics, isolates 1 and 2 were *Mucor* sp. and isolate 3 was identified as *Trichoderma* sp. (Kidd *et al*., 2016; Hurdeal *et al*., 2021). Figures 3 and 4 showed growth colony morphology and microscopic characteristics of *Mucor* sp. and *Trichoderma* sp. The hyphae of *Mucor* sp. appear non-septate, broad and branched [Figure 3(B)]. The colour of the pure culture is greenish [Figure 3 (A)]. Microscopic examination revealed spherical sporangia and subspherical columella [Figure 3 (C and D). *Trichoderma* sp. have septate hyphae, branched conidiophores and brush-like spores at the end of conidiophores when viewed microscopically [Figure 4(B, C, and D)]. The colour of the growing colony was whitish and appeared slightly raised [Figure 4(A)]. The conidia are oval to round and located at the tip of the phialides [Figure 4(D)].

Table 2: Macro-morphology and micro-morphology of fungal isolates

Isolates	Species	Morphology	
		Macro	Micro
Isolate 1	*Mucor* sp.	White to greenish mycelium	Coenocytic hyphae with globose sporangia and sub-globose columellae
Isolate 2	*Mucor* sp.	White to greenish mycelium	Coenocytic hyphae with globose sporangia and sub-globose columellae
Isolate 3	*Trichoderma* sp.	White, thick mycelium Fast growing	Septate hyphae, conidiophore branched, brush-like spore at the ends of the conidiophores and oval conidia at the tip of the phialides



Figures 3: Growth colony morphology and microscopic characteristics of *Mucor* sp. Fungal growth on PDA agar (A), non-septate hyphae100x (B), spores 100x (C) and Sporangium with conidiophore 100x (D)



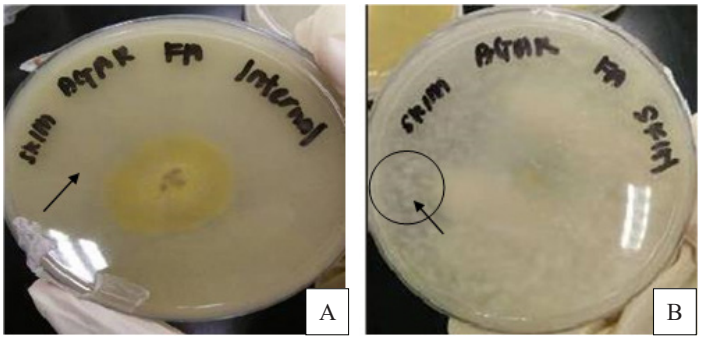
Figures 4: Growth colony morphology and microscopic characteristics of *Trichoderma* sp. Fungal growth from PDA agar (A). Septate hyphae 100x (B), Spores 100x (C) and Sporangium and conidiophore 100x (D)

Screening of Pathogenic Fungi Using Extracellular Enzymatic Test

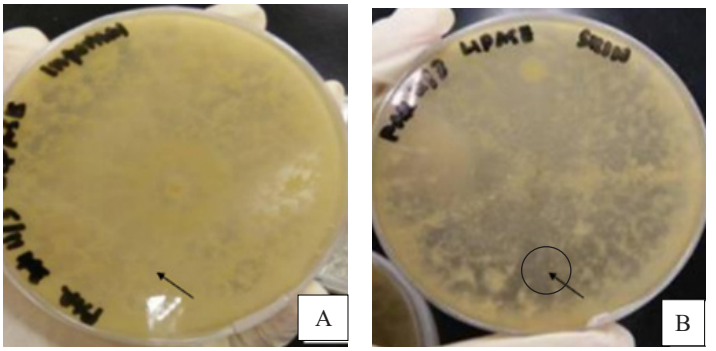
All isolates in the sample were subjected to extracellular enzyme assay. The test was performed to confirm whether the fungi were pathogenic. The test included assays to detect proteolysis, amylase, and lipase (see Table 3). Isolate 1 tested negative for all the extracellular enzymes. Isolate 2 tested positive for amylase. Isolate 3 tested positive for all enzymes. Figures 5 and 6 show the differences between the positive and negative results of the proteolysis and lipase tests. Precise formation on agar was a positive result. Figure 7 shows the difference between the positive and negative results of the amylase test. Precise formation formed around the fungi for a positive result.

Table 3: Results of extracellular enzyme test of proteolysis, amylases and lipases

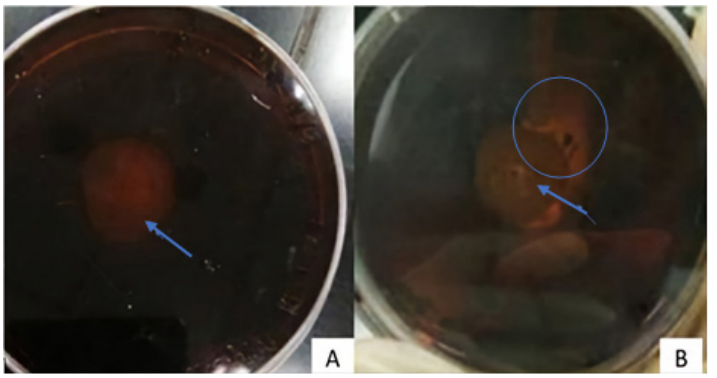
Isolates	Species	Proteolysis Test	Amylases Test	Lipases Test
Isolate 1	*Mucor* sp.	Negative	Negative	Negative
Isolate 2	*Mucor* sp.	Negative	Positive	Positive
Isolate 3	*Trichoderma* sp.	Positive	Positive	Positive



Figures 5: The negative (A) and positive results (B) of the proteolysis test. Clear formation (circle) formed on agar for a positive result



Figures 6: The negative (A) and positive (B) results of the lipase test. Clear formation (circle) formed on agar for a positive result



Figures 7: The negative (A) and positive result (B) of the amylase test. Clear formation (circle) formed around the fungi (arrow) for a positive result

Discussion

Identification of Fungus from Diseased African Catfish

According to the result of morphological identification, three fungi were isolated and identified as *Mucor* sp. (n=2) and *Trichoderma* sp. (n=1). These two species (sp.) are common fungal species that affect African catfish (Melaku *et al*., 2017; Atawodi *et al*., 2018). The results of the macromorphology and micromorphology of *Mucor* sp. and *Trichoderma* sp. can be seen in Table 2. *Mucor* is the most species-rich genus within the Mucorales and is mainly saprobes, endophytes, and parasites of plants and human pathogens causing Mucormycosis (Mendoza *et al*., 2014; Wagner *et al*., 2020). *Mucor* sp. form fast-growing colonies and can be characterized by simple or branched sporangiophores, non-septate, spherical sporangia, encrusting sporangial walls, and zygospores on opposite or tongue-shaped suspensors (Hurdeal *et al*., 2021). As Atawodi *et al*. (2018) noted, *Mucor* sp. causes the highest percentage of infections among African catfish at 49.57%, followed by *Trichoderma* sp. (8%). Melaku *et al*. (2017) found that *Mucor* sp. is the most common fungal species infecting fish and that most fungi are considered opportunistic pathogens. However, some of them may have virulence factors and cause disease. *Mucor* infection can affect the visceral organs and superficial skin of the fish host, resulting in severe disease. The main clinical symptoms in fish are lethargy, imbalance and non-ulcerative dermal lumps while swimming incoherently (Ke *et al*., 2010). *Trichiderma* infection in fish is a relatively rare occurrence. Though *Trichoderma* sp. are mostly not pathogenic fungi to fish, some *Trichoderma* sp. have been isolated from infected African catfish (Diab, 2006; Zakaria *et al.*, 2021) and silver carp (Saleemi *et al.,* 2021). *Trichoderma marneffei* can cause infection of the lungs, liver, skin, lymphatic system, spleen, and bones.

The extracellular enzymatic test was performed to confirm whether the fungi are pathogenic. The test includes a proteolysis assay, an amylase assay and a lipase assay which were performed over a period of one week. According to Abdel-Raheem and Shearer (2002), the absence of positive results may mean that the enzyme is either not produced and not released from the mycelium or that the enzyme is produced and released, but the media hinders its detection. Thus, no positive result does not mean that a species cannot produce a particular enzyme. As mentioned in the results, *Trichoderma* sp. tested positive for the enzymes. However, one strain of *Mucor* sp. showed positive results in the amylase and lipase tests while the other strain of the same species did not show positive results in any tests.

Fungi cause disease outbreaks in various aquatic organisms, including African catfish, which were used for this study. Fungi that cause infections are widespread and distributed worldwide (Siddique *et al*., 2009). Disease outbreaks lead to a decline in overall fish production and are considered a major threat to the commercial success of aquaculture (Melaku *et al*., 2017). Proper water quality control and handling are important to avoid most environmental problems (Idowu *et al*., 2017). Atawodi *et al*. (2017) state that fish condition is affected by water quality and that some fungal species are pathogenic. However, others require environmental stresses such as malnutrition, unstable water temperature, poor water quality and overcrowding. Fungal infections are opportunistic and only affect fish when they are stressed by poor conditions, water quality problems and temperature fluctuations (El-Tawab *et al*., 2020).

Maintaining optimal water quality for the fish species being cultured is a fundamental aspect of successful culture. It is vital to recognize the diagnostic signs of stress and mortality caused by unfavourable physical characteristics of the water in which the fish are kept. For example, inappropriate temperature,

salinity or dissolved oxygen levels are important first steps in disease diagnosis, as these basic parameters should be ruled out before addressing the possible toxicological and biological causes.

Conclusion

In this study, three species of fungi were identified as *Mucor* sp. (n=2) and *Trichoderma* sp. (n=1), isolated from African catfish bought from markets in the area of Kuala Terengganu, Terengganu, Malaysia. The results of this experiment were compared with fungal identification manuals to identify the species. In addition, the result of the extracellular enzyme test showed that *Trichoderma* sp. is more virulent than *Mucor* sp. Finally, one of the factors that cause fungal infection is water quality. Farmers need to monitor water quality daily to prevent fungal infections.

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