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ISOLATION AND SCREENING OF FUNGI ASSOCIATED WITH DISEASED RIVER CATFISH, *Pangasius hypophthalmus*

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Abstract: The production of river catfish *Pangasius hypophthalmus* trade has increased significantly over the past decade. Concerning the high demand for river catfish, *P. hypophthalmus*, these freshwater fish are susceptible to fungi infection that can cause mortality to individual and fish eggs. This study has been conducted to identify the morphology of isolated fungi and their extracellular-enzymes for virulent screening. Three fishes with apparent signs of infection such as ulcerative, haemorrhages and dermal lesions were collected from the fish cage culture at Kampung Serada, Kuala Terengganu. Fungi were isolated from the fish externally (fin, skin, mouth, gill) and internal organs (kidney and liver). The fungal morphology was characterised macroscopically by observing the colony features such as margin, pigmentation (colour), elevation, texture and shape of fungi on an agar plate. Meanwhile, for micromorphology observation were characterised the fungi structure includes the spore, hyphae, and conidiaphore. Based on the identification using a general key to characteristics moulds, two fungi have been classified into two genera: *Rhizopus*-like sp and *Geomyces*-like sp. These fungi proceeded with extracellular enzyme tests such as the proteolysis assay, amylases test and lipases test. The reaction enzymes of fungi from the amylases test showed the clearing zone around the radial colony growth of fungi. The test strain of fungi screened during the present investigation proved to be an efficient producer of protein and polysaccharide degrading enzymes. Rhizopus-like sp and Geomyces-like sp to produce extracellular amylase enzyme indicated the virulent characteristic of the isolate and the ability of those fungi to initiate infection or resist harsh environments and treatments.

Keywords: River catfish, *Pangasius hypophthalmus*, clearing zone, fungi structure, extracellular enzymes, morphological characteristic.

Introduction

Pangasius hypothalamus is one of the most dominant species in the Mekong River. Consequently, it contributes to the largest and most productive inland fisheries globally, mainly to the food source. The traditional development of capture-based aquaculture for this species, particularly in Vietnam and to a lesser extent in Thailand and Cambodia, probably began because of the prolific spawning, producing relatively large numbers of larvae easily harvested from the following river (Poulsen *et al.,*2008).

P. hypophthalmus is the third most cultured freshwater aquaculture species in Southeast Asia, owing to its rapid growth and popularity as a food fish. Although the fish's native habitat is limited to a small geographical region in the Mekong River basin, it has now been introduced to several Asian countries for aquaculture, including Laos, Myanmar, Nepal, Malaysia, and Pakistan, as well as India (Silva and Phuong, 2011).

Despite observing healthy growth, the aquaculture industry, including *P. hypophthalmus* culture in Southeast Asia, is still facing disease problems, affecting its sustainability (FAO, 2001). Infectious and non-infectious diseases have been recorded in farmed *P. hypophthalmus*, from hatcheries to grow-out during the development cycle. In addition, several diseases have been observed, some of which are not caused by pathogens, such as poor nutrition or poor water quality, but many pathogens, such as parasitic, fungal, and bacterial diseases, have

been documented frequently during the culture period.

This situation can also be observed in P. hypophthalmus culture in the Mymensingh area, Bangladesh, conducted from November 2000 to March 2001. The samplings were done monthly, where ten fish from the culturing pond were chosen randomly to be examined for fungal infection. Cotton wool-like lesions on the fish body and ulceration of the skin muscle were found during the period and identified clinically. Two fungi were isolated from *P. hypophthalmus* due to this finding, and they were identified as *Saprolegnia spp.* and *Achlya spp*. (Zahura *et al.,* 2004). This situation was alarming as those aquatic fungi were pathogens isolated in routine isolation without any disease outbreak reported.

A study by Zakaria *et al.* (2020) revealed that food and ornamental fish are susceptible to fungal infection. In this study, a total of 12 fungal were isolated from 4 ornamental fishes, including diseased gold gourami (*Trichopodus trichopterus*), snakeskin gourami (*Trichogaster pectoralis*), angelfish (*Pterophyllum scalare*), African sharptooth catfish (*Clarias gariepinus*) and one food fish, red hybrid tilapia (*Oreochromis* spp.) with clinical sign of dermal lesions and erratic swimming behaviour. The result revealed that they are generally opportunistic invaders but are often fatal and hard to treat once established. Therefore, fungi and straminipilous species in an aquaculture environment may be problematic pathogens under stressful conditions (Leano, 2001).

Thus, the present study would like to isolate fungi associated with the disease river catfish, *P. hypophthalmus* and investigate the virulent properties of fungi using extracellular enzyme production of isolated fungi.

Materials and Methods

Sample collection

Fishes of *P.hypophthalmus* showed clinical signs of infection such as ulcerative, haemorrhages, and dermal lesions collected from a fish farm located at Kampung Serada, Kuala Terengganu (5.280129632449689, 103.08463466930846). This place is a fish cage culture place in the Terengganu area that widely supplies freshwater fish to restaurants and markets. Three fishes with obvious clinical signs of infection were placed in a container and transported back to the Aquatic health organism lab (MKOA) of the University Malaysia Terengganu for further examination, where they were kept alive using portable aerators.

Isolation of fungi

Fungi were isolated from the external part of fish that showed obvious clinical signs and for internal organs, kidneys and liver were chosen despite non-distinct differences. Each external area was swabbed using a sterile cotton bud and placed on potato dextrose agar (PDA) agar individually in a zig-zag design. Meanwhile, fungi from internal organs were isolated by the dilution method. Each internal organ was crushed in 1 mL of sterile distilled water to make serial dilutions (10^{-1} to 10^{-3}). One hundred µL of final dilution was placed on PDA plates composed of 20 g dextrose, 15 g agar, and 4 g potato starch mixed with 1 L containing 200 µg/mL of chloramphenicol to remove contamination from bacteria. The agar plates were incubated at 25°C for 5 days. Finally, an agar block cut from the edge of each fungal colony was transferred to a new PDA agar plate to establish a pure culture (Lee *et al.,* 2016).

Identification of isolated fungi

The fungal morphology was characterised macroscopically by observing the colony features such as margin, pigmentation (colour), elevation, texture and shape of fungi on an agar plate meanwhile for micromorphology observation was characterised using a light microscope (Olympus, Japan) with the application of one drop of lactophenol cotton blue as a staining agent on the glass slide with a piece of mycelium. Fungi structure includes the spore, hyphae, and conidiaphore were observed. The isolated fungi were identified based on macromorphology and micromorphology references at the National mycology reference

centre, University of Adelaide (https://mycology.adelaide.edu.au/reference-centre/).

Production of extracellular enzyme

Fungal enzymes play an important role in the pathogenicity of the infection. Hence, extracellular enzyme tests were conducted to identify the enzyme production of proteases, amylases and lipases.

The proteolysis test prepared skimmed milk agar by adding 10 g/Lskimmed milk powder (Sunlac) in PDA. The agar and skimmed milk solution were autoclaved separately before sterile mixing. The amylases test was performed using starch agar medium (peptone,0.5g; beef extract, 0.15g; yeast extract,0.15g; NaCl, 0.5g; starch, 1; agar,2g; distilled water, 100ml) and the lipases medium was prepared using 0.3% yeast extract, 0.5% pepsin and 1.0% agar for lipolytic assay. All the mediums were autoclaved at 121°C (250 °F) for around 15–20 minutes. Then the fungi were inoculated in the middle of the agar plate and incubated at 25°C for 2- 3 days.

During the culture period, it was ensured that fungi did not cover the agar plate completely to see the clearing zone formation when flooded with an aqueous solution of 40% ammonium sulphate and 10% Iodine solution for protease assay and amylases assay, respectively. A clearing zone formation around fungal colonies indicated the presence of the targeted enzyme. For lipases assay, the occurrence of clearing zone can be observed during the fungal growth on agar medium.

Results and discussion

This study was carried out to use the morphology characterisation method to identify fungi isolated from diseased *P.hypophthalmus* from a fish cage culture area in the Terengganu area.

Fish sample

The present studies sampled the affected fishes based on their appearance and apparent clinical sign. In addition, three river catfish with clinical signs of haemorrhages at the mouth area, pectoral fin and caudal fin (Figure 1) were collected for further fungal isolation. Isolated fish exhibited weak behaviour during transportation and prior to dissection and a lack of aggression. The average size of three river catfish was 1.23 kg and 38 cm for body weight and total length.

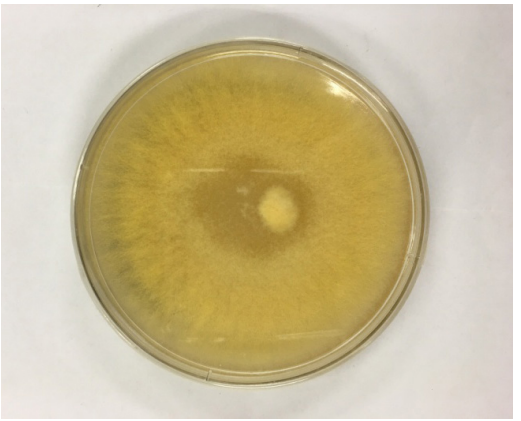
Figure 1: Obvious lesions on affected fishes. A) Haemorrhages observed at mouth area and pectoral fin of Fish 1. B) Haemorrhages were observed at the mouth area of Fish 2. C) Reddish area was also spotted at the tip of the pectoral fin of Fish 3. Clinical signs were shown with a white arrow

Morphological identification of fungi

The isolated fungi were examined in this study based on cultural, microscopic and morphological characteristics. Figure 1 and 2 show two fungal species isolated and identified in this study. Out of three fishes collected from fish cage culture, Fish 1, which has clinical signs of haemorrhages observed at the mouth area and pectoral fin exhibits the most severe clinical signs; hence two fungal were both isolated from this fish.

Two fungi were isolated from Fish 1 and no growth of fungi was observed from the media of Fish 2 and 3. Macromorphology and micromorphological analysis identified the isolates presumptively to at least at genus taxon. However, further micromorphology observation and molecular identification must be done to confirm the genus and species.

The colony morphology of *Rhizopus*-like species shown in Figure 2 reveals a creamy white colour colony, curled margin, convex elevation dry textured and rhizoid shape on the top of agar (A1). Observation under the microscope showed septate hyphae with an elliptical shape of spore.



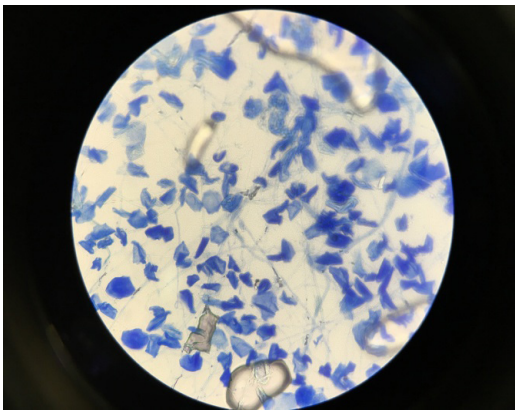
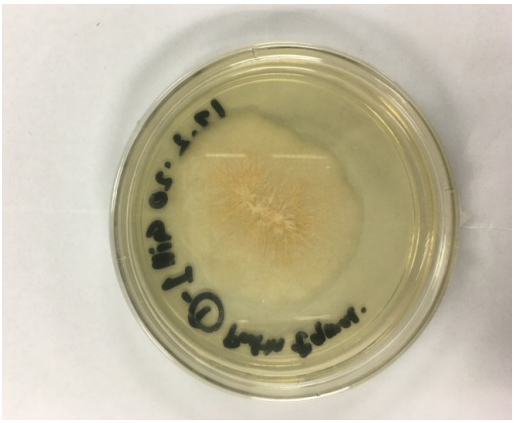


Figure 2: *Rhizopus*-like species features on PDA (A1, top) and spore (A2) under 40X magnification

The velvety white colour surface, undulate margin, convex elevation, dry texture and filamentous shape were characterised on the top of agar (B1) of *Geomyces*-like species culture. In addition, burst sporangium with subglobose spore and septate hyphae were micromorphologically observed (B2), as shown in Figure 3.



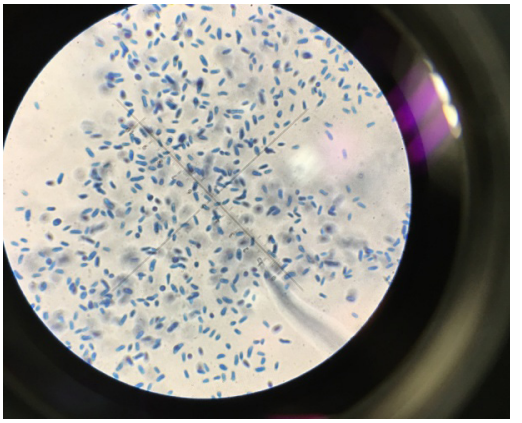


Figure 3: *Geomyces*-like species features on PDA (B1, top) and spore (B2) under 40X magnification

Origin of fungal isolates

The distribution of the isolated species from different fish parts is shown in the table. Fungi were isolated from the external part of fish (fin, skin, mouth, and gills) and internal organs (kidney and liver). The result showed that both *Rhizopus*-like and Geomyces-like species were isolated from Fish 1 in gills and kidney areas. The isolated species belong to the family Rhizopodaceae (*Rhizopus*-like *sp*) and family Myxotrichaceae (*Geomyces*-like *sp)*.

Table 1: Distribution of isolated species at different fish part

Fish	Species/fish part	External part				Internal part	
		Fin	Skin	Mouth	Gills	Kidney	Liver
Fish 1	*Rhizopus*-like species	-	-	-	-	+	-
	Geomyces-like species	-	-	-	+	-	-
Fish 2	-	-	-	-	-	-	-
Fish 3	-	-	-	-	-	-	-

Rhizopus sp also has been linked with frequently isolated fungi from contaminated fish feeds and feed ingredients in Kenya (Njagi, 2016), Brazil (Gonçalves-Nunes, 2015) and Egypt (Hassan *et al*., 2011) probably the presence of this fungi is linked with fish feed from fish cage culture in Terengganu area. However, this species also has been reported to contribute to fish disease. A study by Haroon *et al.* (2014) revealed that *Rhizopus* sp were isolated from different organs such as at external and internal fish body of *Carassius auratus*, *Xiphophorus maculatus* and *Poecilia reticulata* with clinical signs of eroded scales, haemorrhages, lesions, whitish gills and damage of pectoral and pelvic fins. A similar clinical sign of haemorrhages with current studies was observed even though the condition is not severe.

Meanwhile, *Geomyces* sp is a member of Ascomycota, also known as sac fungi. These species are generally found in relatively cool habitats and can adapt to various climate conditions (Johnson *et al.,* 2013), making them able to survive in various conditions and play a big role in pathogenicity. Furthermore, the ability of Ascomycota to switch from non-pathogenic to pathogenic has long been recognised (Klein & Tebbets, 2007).

Extracellular enzymes test

From the extracellular enzymes test (Table 2), none of the fungi showed a clearing zone after applying ammonium sulphate on agar for proteolysis and lipases test on lipases medium. The negative result indicated that the fungus does not produce the enzymes. Meanwhile, all the fungi showed a clearing zone for the Amylases test (Figures 3 and 4).

Table 2: Result of extracellular enzyme test of proteolysis, amylases and lipases

Fungi	Proteolysis	Amylases	Lipases
A	Negative	Positive	Negative
B	Negative	Positive	Negative

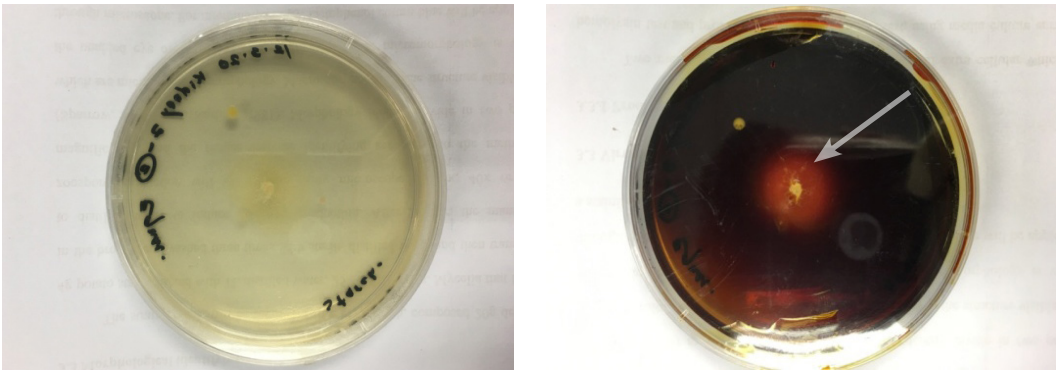


Figure 4: Amylases test of *Rhizopus*-like species before (A) and after (B) flooded with iodine solution on starch agar medium. Clearing zone showed with a white arrow

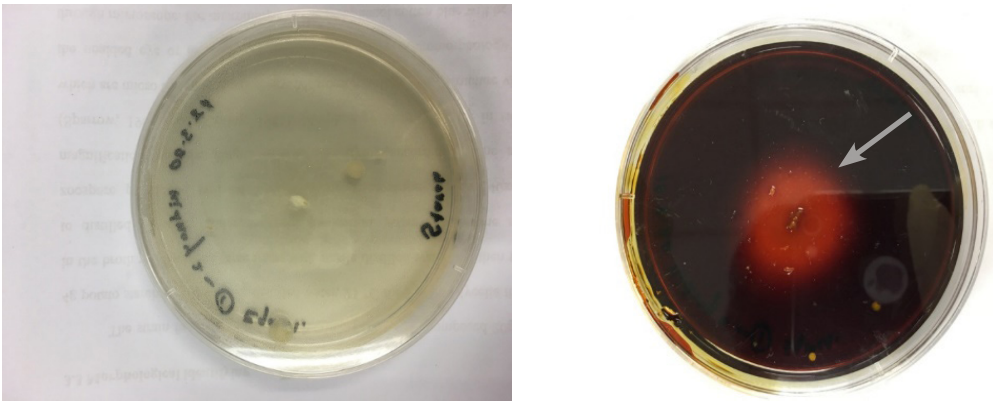


Figure 5: Amylases test of *Geomyces*-like species before (A) and after (B) flooded with iodine solution on starch agar medium. Clearing zone showed with a white arrow

This extracellular enzyme activity forms a clear zone, allowing the organism to consume protein from the agar (Balaji *et al*., 2012). In addition, the lipolytic activity demonstrates the ability of the fungi to hydrolyse lipids (Furumura *et al*., 2006). Proteolytic enzymes can cause massive tissue damage in the host, which facilitates an infection (Gunnlaugsd´ottir and Gudmundsd´otter, 1997). Protease can cleave peptide bonds and functions in the pathogenesis and virulence of specific illnesses (El-Barbary, 2010). Meanwhile, for amylase activity that involve polysaccharide degrading enzymes, the production of this enzyme often relates to the ability of the organism to protect itself from being killed by various environmental condition (John *et al.*, 2019) and also able to degrade other biofilm-forming organisms (Elamary and Salem, 2020).

Conclusion

Fungi that involve in virulence and pathogenicity of infected fish show successful adaptation in the aquatic environment. The present study was conducted to isolate, identify and characterise extracellular enzyme production of fungi isolated from *P.hypophthalmus*. Two fungi were successfully isolated: genera *Rhizopus*-like sp and *Geomyces*-like sp. However, further micromorphology observation and molecular identification must be done to confirm the genus and species. Rhizopus-like sp and Geomyces-like sp to produce extracellular amylase enzyme

indicated the virulent characteristic of the isolate and the ability of those fungi to initiate infection or resist harsh environments and treatments.

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