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The first report of *Aspergillus ochraceopetaliformis* Bat. & Maia from small indigenous dry fish *Setipinna phasa* (Hamilton 1822) in Bangladesh

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Abstract

Setipinna phasa is known as Phasha maach, a common fish in Bangladesh popular as sutki (dried fish). Since Bangladesh exports a significant amount of dried fish to other countries, this study was conducted to find out the fungus associated with the dried fish as well as its morphological characteristics, cultural factors and molecular identification. *Aspergillus ochraceopetaliformis* was isolated from *Setipinnaphasa* and identified by analysis of mycelium and conidia morphologyas well as by analysis of the internal transcribed spacer region (ITS) of *Aspergillus ochraceopetaliformis* PCR products measuring 606 bp and a blast search revealed 99% sequence similarity to the *Aspergillus ochraceopetaliformis* species complex. Analysis of the pathogen's vegetative growth characteristics revealed that Hennerberg media (22mm) and yeast extract mannitol media (12mm), had, on average, the highest and lowest mycelial growth rates, respectively. The optimal pH and temperature for mycelial growth of *Aspergillus ochraceopetaliformis* were 7 and 30˚C, respectively. To the best of our knowledge, this finding of *Aspergillus ochraceopetaliformis* from *Setipinna phasa* is the first-ever work in Bangladesh.

Key words: *Aspergillus ochraceopetaliformis*, Culture media, Dry fish fungus, Growth characteristics, Molecular identification, *Setipinna phasa*.

INTRODUCTION

The Gangetic hairfin anchovy or *Setipinna phasa* (Hamilton, 1822) fish are carnivores in nature and mostly devour the food found on the water's surface and in the water column in their habitats. According to Shafi & Quddus (1982), the fish is widespread in the tidal rivers, estuaries and shallow coastal regions of Bangladesh, as well as in former Burma (Myanmar) and undivided India (Day, 1889). *S. phasa* have been regarded as an excellent source of important proteins, macronutrients and micronutrients, vitamins and minerals, all of which can significantly contribute to meeting a person's nutritional needs. These minerals are necessary for building disease resistance in the body. The various minerals sodium, potassium, calcium, iron, zinc and manganese are present in significant ratios. Consuming these fish species can have a positive impact on the country's health in a country where the population suffers from malnutrition and protein deficiency (Masud *et al*., 2023).

Preserving fish is essential because spoilage occurs so quickly. There are numerous fish preservation techniques around the world. In Bangladesh, solar drying is the most popular and cost-effective method (Sultana *et al*., 2020). Dried fish is locally called Shutki. In

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Bangladesh, Shukti is a favorite dish. According to Ara *et al*. (2020), Bangladesh exports large quantities of dried fish, which generates significant foreign exchange earnings.

Numerous fungi include *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Rhizopus* spp., *Mucor* spp., *Acremonium* sp., *Wallermia seba and Sporodendrone pizoum* are isolated from dried fish in Bangladesh, Hong Kong, India, Malaysia, Pakistan, and Thailand. Various fungi especially *Aspergillus* species, are known to produce various toxins that can be harmful human health. These toxins include aflatoxins, ochratoxins and sterigmatocystine (Motalebi *et al*., 2008). These significantly reduces the consumption of dried fish. According to Sivaraman *et al*. (2018), halophilic molds were discovered in salted shukti. These molds included *Basipetosporahalophila* (Syn: *Oonsporahalophila*), *Polypaecilum pisce*, *Eurotiumams telodami*, *E. repensan, E. rubrum*, *Penicilliumcitrinum*, *Aspergillusflavus*, *Aspergillus niger*, *Aspergillus sydowii*, *Aspergillus wentii* and *Aspergillus penicilloides* (Chanda *et al*., 2021). Furthermore, *B. halophilica* in Veravel (Gujarat, India), *Aspergillus*, *Rhizopus*, *Penicillium*, *Absidia* and *Mucor*, were the most abundant fungal genera. When pathogenic fungi such as *Aspergillus* sp., *Lichtheimia* spp. *And Curvularia* spp. are present, the handlers and consumers may develop aspergillosis, mucormycosis and phaeohyphomycosis.

There are few reports on the related fungi found in dried fish from Bangladesh. In dried fish, the presence of fungi signals the beginning of decomposition and product breakdown. Again, fungi can produce harmful mycotoxins that can be mutagenic to human health under certain conditions. The current study will provide detailed information about the occurrence of fungi in *S. phasadry* fish, which would be essential for the implementing of appropriate and effective measures for the production, storage and transportation of high-quality dried fish. Therefore, the aim of the current experiment was to identify and study the morphology, biology and culture conditions of dry *Setipinna phasa* fish using traditional fungal taxonomy and ITS sequence analysis.

MATERIALS AND METHODS

Collection and preservation of samples: Phasa fish samples were collected from Baipail Bazar, Gazipur and brought to Bangamata Begum Fazilatunnesa Mujib Hall of Jahangirnagar University to prepare these samples as dried fish. These fresh samples were processed in three ways. First, some of the samples were washed with normal water and dried, in the sun, next, the washed sample were mixed with salt and dried in the sun, and finally the remaining washed samples were dried in the sun with salt and turmeric powder. Dried fish samples were preserved in separate jars. These were immediately taken to the Laboratory of Mycology and Plant Pathology, Department of Botany, Faculty of Biological Science, Jahangirnagar University, Savar, Dhaka for isolation and identification of dry fish fungi.

Isolation of the fungus: Visible fungal growth on the prepared dry fish was observed. The fungus was isolated through the tissue-planting method. In each piece of the tiny, 0.5 cm-long infected sections, tissues from both fungus-infected and uninfected dried fish parts were combined. These tiny pieces were rinsed several times with distilled water and

these pieces were placed in a 90mm petridish with potato dextrose agar (PDA) medium without any sterilization and stored in a 12°C environment for ten days at pH 7 and a temperature of 25°C bred/12 dark/ light and the fungal colonies were observed. Mycelia from growing fungal colonies were transferred to fresh PDA plates to obtain a pure culture. After several transfers of the hyphal tip, a pure culture was created.

Morphological identification of the isolated fungus: The pure culture of the isolated fungus was identified microscopically using standard methods (Ahmmed *et al*., 2022). According to Seifert (1995), microconidia and macroconidia were observed in 10 days old culture and classified based on the color of the conidial masses, the shape, septation and basal and apical cell of the macroconidia, the shape of the microconidia, conidiophores in the aerial mycelium and the presence or absence of chlamydospores.

Molecular characterization: Molecular characterization was done with the help of commercial service provided by Invent Technology, Dhaka, Bangladesh. Fungal genomic DNA samples were extracted using the Maxwell Cell Kit (AS1030, Promega, USA). The primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAA AGTCG TAACAAGG-3′) were used for the PCR reaction (Alam & Rahman, 2020). The PCR reaction was performed with 20 ng of genomic DNA as the template in a 25-µl reaction mixture having an LA Taq (TAKARA BIO INC, Japan). The thermal cycle was performed with activation of Taq polymerase at 94°C for 1 minute; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 5 minutes each; finishing with a 10-minute step at 72°C for final extension. Amplified PCR products were electrophoresed on 1.5% agarose gel in 1 × TAE buffer for 1hr at 100V with a 1kb DNA ladder as a size marker and then stained while agitated in an EtBr solution (0.5%µg/mL). The stained gels were visualized and photographed using a UV transilluminator (Kodak Image Station 4000R; Molecular imaging system, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608). The Maxwell® 16 DNA Purification Kits were used to purify the amplification products (Promega, USA). The purified PCR products were sequenced bi-directionally in First BASE Laboratories SdnBhd (Malaysia).

DNA sequences were checked by Bio Edit and MEGA 11. A BLAST search with the ITS sequences was used to reveal the closest matching taxa. Multiple sequence alignments were done using MEGA6. Data were converted from Fasta to MEGA format using Clustal W. The evolutionary models were determined using the Akaike Information Criterion (AIC). The Tamura-3 parameter was selected as model for the analysis. Maximum likelihood (ML), neighbor-joining (NJ) and maximum parsimony (MP) analyzes were performed and the robustness of the branches was determined using 1000 bootstrap replicates along with max-trees set to 1000. The number of replicates was derived using stopping criterion. Bootstrap values greater than 60% were accepted (Tamura *et al*., 2013).

Effect of culture media, temperature and pH on the vegetative growth of the studied fungus: Five distinct culture media, including Potato dextrose agar (PDA), Yeast extract mannitol (YEM), Glucose peptone (GLP), Hennerberg (HEN) and Hoppkins (HOP) media, were utilized to assess the mycelial growth characteristics of the isolated fungi. The effect of pH on the growth of the pathogen was assayed on the PDA medium.

Different pH levels *viz.*, 6.0, 7.0 and 8.0 were used. Before autoclave the medium was adjusted to pH 6, 7 and 8 with the addition of 1 N NaOH or HCl and it was incubated at 30ºC for 10 days. The radial growth of mycelia on each petri dish was measured in 3 directions.

Statistical analysis: The data generated during the research activity was analyzed using industry-standard statistical analysis programs like MS Excel, SPSS 16.0, MEGA 11.0 and BLAST. In SPSS-16, one-way ANOVA and Duncan's post-hoc analysis were used to analyze the data.

RESULT AND DISCUSSION

Dry fish fungus of *Setipinna phasa*: Dried fish fungus, *Aspergillus ochraceopetaliformis* was isolated from the selected indigenous species of fish, *Setipinna phasa*.

Morphological characterization of isolated fungus: The *A. ochraceopetaliformis* colonies expand quickly (45 to 55mmin 7 days). While the conidial heads are normally distributed in zones on an agar plate, the vegetative mycelium is mainly immersed in the agar. The colony's distinctive color is yellow. Some *Aspergillus sp.* colonies produce pinkish to purple, amorphous, pebble-like sclerotia. On a petri dish, the reverse side appears pale to brownish. The conidiophores of *Aspergillus sp.* resemble a powdery mass to the unaided eye. Phialides are placed on the conidial heads in a biseriate pattern under a microscope, either smooth or finely roughened (i.e., phialides are attached to intermediate cells called metulae, which in turn are attached to the vesicle). The metulae grow radially around the edge. Initially globose in culture, the conidial heads eventually acquire two or three divergent columns as the conidial chains stick to them over time. Chalky yellow to light yellow-brown is the distinctive color of conidiophores. These conidiophores have granular, pale yellow- brown walls and abrupt attachments to "globose to subglobose vesicles.

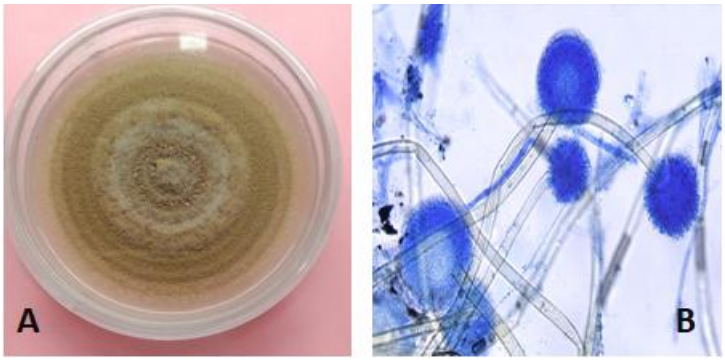

Fig. 1. Morphological characteristics of *Aspergillus ochraceopetaliformis*. A. Vegetative growth of *A. ochraceopetaliformis* in PDA medium; B. Microscopicview of conidia, conidiophore of *A. ochraceopetaliformis*

Molecular characterization of isolated fungus: Using ITS1 and ITS4 primers, the 606 bp ITS region was amplified and sequenced (Fig. 2). The internal transcribed spacer (ITS) region of genomic DNA has recently been found to be extremely helpful for identifying fungus at species levels, according to molecular phylogenetic research. When it comes to variation between species and even strains, the internal transcribed spacer of rDNA is thought to beakey factor (Alam *et al*., 2010).The PCR yielded an amplicon size of about 606 bp during the amplification of the ITS region of *Aspergillus ochraceopetaliformis.*

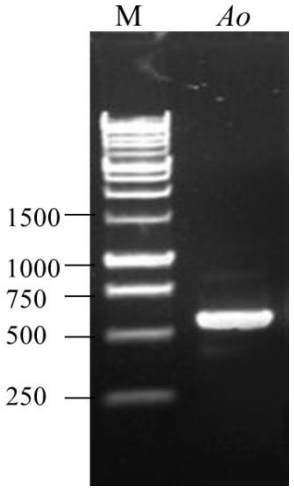

Fig. 2. Profiles of amplification of rDNA-internal transcribed spacer region of the tested fungus. M. molecular size marker (1kb DNA ladder); *Ao*: *Aspergillus ochraceopetaliformis*

The BLAST search analysis revealed that our organism *Aspergillus ochraceopetaliformis* (study sample) has 99% identity with other *Aspergillus ochraceopetaliformis* (OP237490.1), KF384187.1 (*A. ochraceopetaliformis*), MH857406.1 (*A. ochraceopetaliformis*), OW9888382.1 (*A. ochraceopetaliformis*), MH141440.1 (*A. ochraceopetaliformis*) etc. According to the results, each unique species of *A. ochraceopetaliformis* belongs to a single cluster. According to Alam *et al*. (2010), ITS sequences are genetically stable or exhibit minimal variation within species, but differ between species within a genus. From the available facts it is clear that the fungus we examined is *A. ochraceopetaliformis*.

Nineteen fungal species were retrieved from the NCBI database and a phylogenetic tree was built based on the nucleotide sequences of the ITS sections. The studied sample's ITS region rDNA sequence was compared to *Aspergillus ochraceopetaliformis*, a previously recognized fungus, using accession number H109740.1 to determine the percentage of homology. Six different clades were identified in the phylogenetic tree using neighbor-joining methods (Fig. 3). The sequences of the ITS region displayed reciprocal homologies ranging from 35 to 99%. The sequencing data of the selected NCBI GenBank strain (MH867670.1 *Agaricus bisporus*) was used as an out group for the comparative on phylogenetic relationships studies with the selected strain of *Aspergillus ochraceopetaliformis* (studied sample).

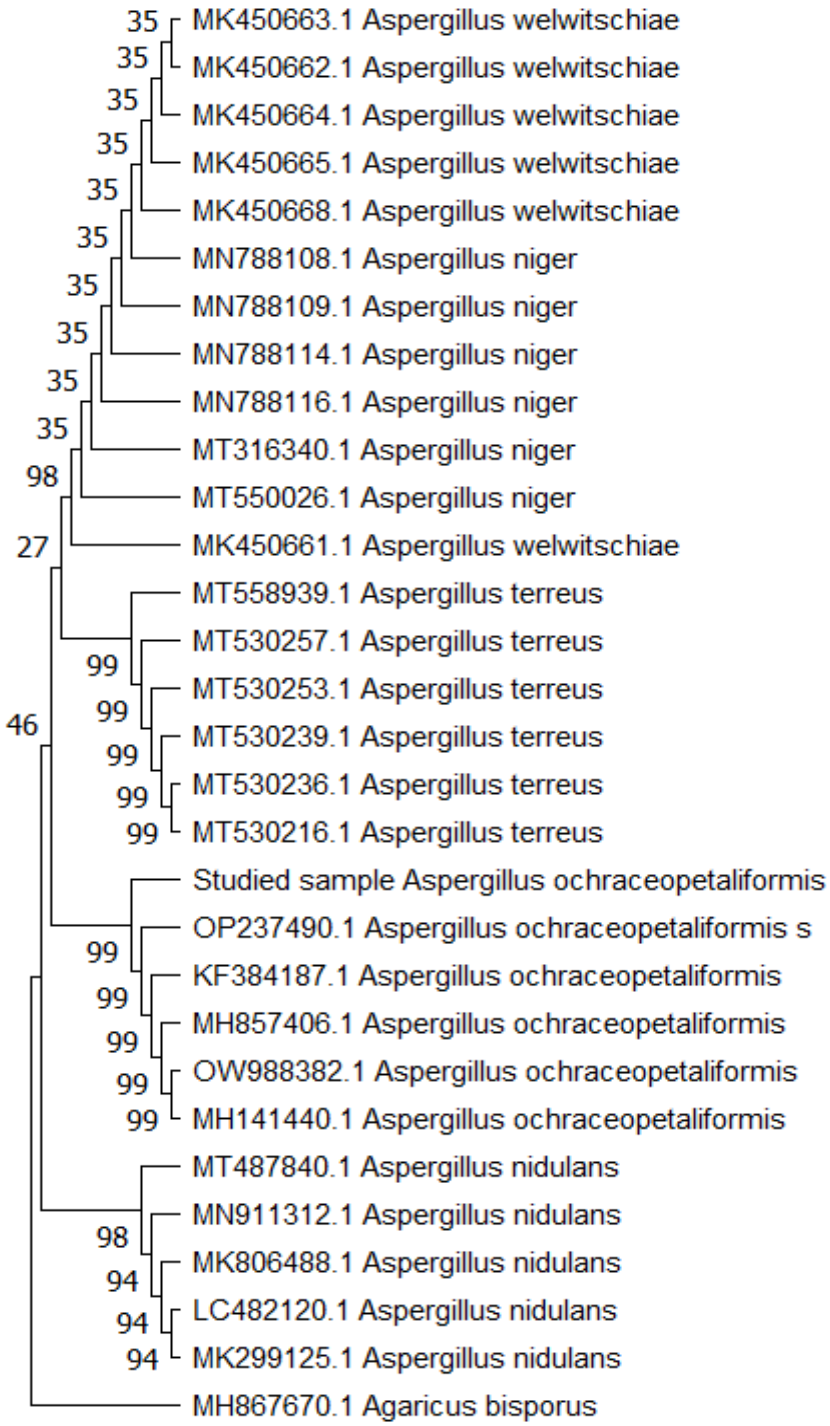

Fig. 3. Neighbor-joining tree of *Aspergillus ochraceopetaliformis* from analysis of ITS sequence with bootstrap value. Our organism is marked as the studied sample

Effect of culture media: The presented study determined the best culture medium for mycelial growth of the selected fungus *A. ochraceopetaliformis* by evaluating five different culture media. The results of the mycelial growth tests show that the Hoppkins (HOP) and Yeast Extract Mannitol (YEM) media were the least favorable, while the Hennerberg (HEN), Glucose Peptone (GLP) and Potato Dextrose Agar (PDA) media were slightly favorable. In Hennerberg media (22mm) and yeast extract mannitol media (12mm), the highest and lowest mycelial growth rates were found after 10 days of incubation. Similar results also supported by Sultana *et al*. (2023). Chanda *et al*. (2021) reported that the mycelial growth pattern of *Aspergillus elegans* was the best on PDA medium.

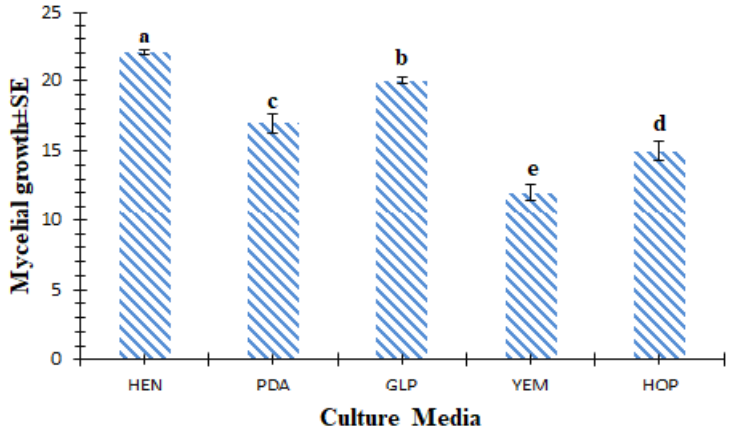

Fig. 4. Effect of different media on the mycelial growth of *Aspergillus ochraceopetaliformis* after 10 days of incubation at 25°C. GLP: Glucose peptone, HEN: Hennerberg, HOP: Hoppkins, PDA: Potato dextrose agar, YEM: Yeast Extract Mannitol

Effect of temperature: Temperature effects on the mycelial growth of *Aspergillus ochraceopetaliformis* on PDA media were evaluated at five different temperatures such as 15°C, 20°C, 25°C, 30°C and 35°C(Fig.5). The data informed that the highest growth of *Aspergillus ochraceopetaliformis* was recorded at 30°C, followed by 25°C. In our experiment, *Aspergillus ochraceopetaliformis* grew maximum at 30ºC which is consistent with the previous findings of Iwen *et al*. (2007) who cited that the highest mycelia growth and sporulation of *A. elegans* registered at 30°C.

Effect of pH: Fungal ecology can be more easily detected by evaluating the pH of the associated fungus. However, three different pH levels viz., 6, 7, and 8 were used to determine the ideal pH for mycelial development. Mycelium developed radially at the fastest place at pH 7 (79mm) but growth was slightly slower at pH 6 (52mm). Our findings were supported by Sikder *et al*. (2019) who stated that the ideal pH of *Aschersonia* sp. for vegetative growth is pH 7.

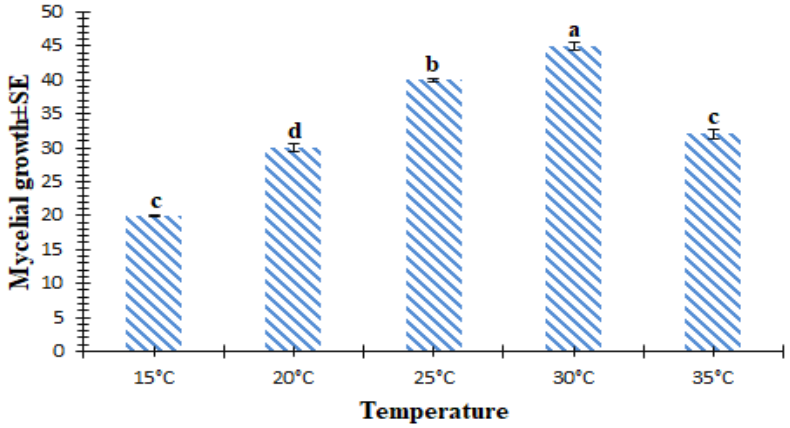

Fig.5. Effect of different temperatures on the mycelial growth of *Aspergillusochraceopetaliformis* after 10 days of incubation at PDA medium

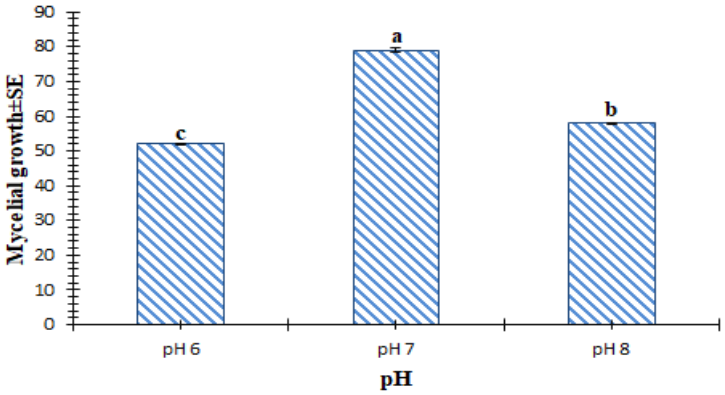

Fig. 6. Effect of different pH on the mycelial growth of *Aspergillus ochraceopetaliformis* after 10 days of incubation at PDA medium

Dried fish such as *Setipinna phasa* is an excellent source of vitamins, minerals and protein and has a high market value in our country. The current research result identifies *Aspergillus ochraceopetaliformis,* a pathogenic fungus from dried fish. Identification of *Aspergillus ochraceopetaliformis* was done by both morphologically features through mycelium, conidia study; and by molecular techniques used the sequence of the ITS region and showed 99% sequence similarity with several Genbank data of *Aspergillus ochraceopetaliformis* species. Under different culture conditions, the vegetative growth characteristics of the pathogen showed that Hennerberg media (22 mm) and yeast extract mannitol media (12mm), had the highest and lowest mycelial growth rates. The optimal pH and temperature for mycelial growth of *Aspergillus ochraceopetaliformis* were 7 and 30°C, respectively.

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