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Morphology and Molecular Identification of Dry Fish Fungus *Cunninghamella blakesleeana* from Small Indigenous Fish "Kachki" *Corica soborna* (Hamilton 1822) in Bangladesh

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Abstract

The present experiment was conducted to investigate a dry fish fungus, *Cunnighamella blakesleeana*, which was identified from the infected part of the *Corica soborna*, locally named as Kachki fish. Mycelium was hyaline, often with granular content, and conidiophores were erected, with verticillate or solitary branches. Zygospores were globose, tuberculate, suspensors equal, smooth, hyaline and heterothallic. Using ITS4 and ITS5 primers, the 740 bp-long ITS region was amplified and sequenced. The ITS region sequences had reciprocal homologies of 98% to 100%. The findings showed that several species of *C. blakesleeana* fall into the same cluster. It has been determined by molecular data that the fungus we had studied was *C. blakesleeana*. The maximum mycelial growth (95.33 mm) was observed in the PDA medium, followed by the PSA medium, and the lowest growth (65.50 mm) was measured in the HPA medium in the study of the impact of culture media on the mycelial growth of *C. blakesleeana*. The influence of temperature on the radial mycelial growth of *C. blakesleeana* on PDA medium was investigated through five different temperatures. Although pH is a crucial factor in understanding the ecology of spoilage fungus, the highest mycelial growth of *C. blakesleeana* (88.25 mm) was seen at pH 7, followed by pH 8 and pH 6, while pH 9 was revealed to have the lowest mycelial growth. The outcome suggested that *C. blakesleeana* thrived in neutral environments.

Keywords

Culture Media, Molecular Identification, Temperature, pH, Vegetative Growth

1. Introduction

Corica soborna is regarded as one of the most well-known small indigenous species (SIS) fish in Bangladesh [1]. According to Craig *et al.* [2], Bangladesh has a highly rich aquatic biodiversity, consisting of 260 native freshwater bony fish species that are fit for human consumption and belong to 145 genera and 55 families. It is rich in vitamin A and vitamin D, both of which are necessary for healthy bones, teeth, skin, and eyes in humans. SIS also offers a sizable quantity of calcium, phosphorus, iron, iodine, and other nutrients. These fish have been regarded as a superb source of vital protein, macro and micronutrients, and minerals, which may significantly contribute to the satiation of a human's nutritional shortfall [3]. Fish is an awfully perishable food item and needs preservation for future uses [4]. One of the widely consumed foods in Bangladesh is dried fish. The most popular technique of fish preservation globally is sun drying [5]. Due to the use of low-quality raw fish for drying, conventional drying techniques, unhygienic and improper sanitation facilities, and sporadic use of unauthorized chemicals and insecticides at various stages of fish drying, Bangladeshi dried fish and fishery products have recently perceived a decrease in export market demand [6].

According to Gupta and Samuel [7], at all humidity levels over 70% where the equilibrium moisture content for these fish was 25%, mold development was a substantial contributor to deterioration. On dried fish, fungus development signals the beginning of decomposition and product degradation [8]. Atapattu and Samarajeewa [9] reported that the most prevalent fungus such as *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. restrictus*, *Aureobasidium* spp., *Basipetospora halophila*, *Cladosporium herbarum*, *Gliomastix*, spp., *Penicillium chalybeum* and *Penicillium expansum* were recorded in dried fish. On dried fish, fungi generate unpleasant tastes, weaken the meat, and in some cases, can produce mycotoxins [10].

Sequence-based identification must be able to distinguish between a range of microorganisms by recognizing a molecular target that is big enough to do so. The rDNA gene complex, found in every microbial pathogen, is one such target area that has been identified. Three genes: 18S, 5.8S, and 28S and two intergenic spacers: ITS1 and ITS2 are found in fungi [11]. There is a great scope and need for conducting a comprehensive study on the molecular characterization of the dry fish fungus *Cunninghamella blakesleeana* from SIS fish. Therefore, the present research work has been undertaken to isolate and identify fungus from small indigenous dry fish *C. soborna* using classical fungal taxonomy and molecular technique. Besides, the effects of selected fungal culture media and two environmental conditions (temperature and pH) on the isolated and identified fungus were also assessed.

2. Materials and Methods

2.1. Fresh and Dried Fish Samples Collection

Corica soborna were collected from different regions of Chalon Beel (Vast water

body) both in fresh and dried (Figure 1) condition, the Chalan Beel is located in the North-West region of Bangladesh, approximately between latitudes 24˚12' and 24˚50' North and longitudes 88˚21' and 88˚35' East in the South-East portion of the greater Rajshahi and Pabna districts.

2.2. Isolation and Identification of Fungi through Morphological Characterization

Dried fish fungus was isolated using a standard blotter and tissue planting method. To detect the fungus; infected dry fish were incubated on three-fold moist blotter discs placed on disposable transparent plastic Petri plates. Three folds of blotting paper of 90 mm size were saturated with distilled water and sited in 90 mm disinfected Petri plates after draining excess water. Petri plates, blotters and distilled water were sterilized before use. The infected dry fish portion is then cut off with scissors and kept in the Petri plate which contains moist blotter discs. Then, the Petri plates were incubated at 25˚C ± 2˚C for seven days under alternating cycles of 12 hours of darkness and 12 hours of daylight. After 7 days of incubation, the appeared fungi were observed under a stereo binocular microscope. For proper identification of fungus, semi-permanent slides were also prepared and observed under a compound microscope. The fungus from the incubated dried fish samples was also transferred to the PDA medium when needed. A suitable portion of the culture of the isolated fungus either from the fruit surface or from PDA plates was selected under a stereoscopic microscope and was taken out with the help of forceps and needles and put in one drop of lactophenol on the cleaned slide. It was then gently warmed by heating and cooling over a low flame of the spirit lamp 6 to 8 times but was never allowed to boil. The material was stained with a small quantity of cotton-blue. A clean cover glass was then placed over the material; excess fluid was removed by soaking it with tissue paper and examined under a compound microscope. The photograph was taken using a photographic microscope. Based on colony morphology, morphological features of conidia, and conidial measurement using standard guidelines, isolated fungi from the diseased tissues of dried fish were identified [12].

2.3. Molecular Characterization

The Maxwell Cell Kit (AS1030, Promega, USA) was used to extract samples of fungus genomic DNA. The PCR reaction was conducted using the primers ITS4

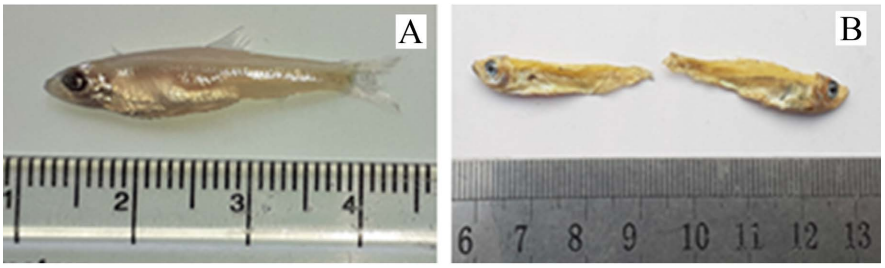

Figure 1. Kachki (*Corica soborna*) in fresh (A) and dried (B) condition.

(5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAA AGTCG TAACAAGG-3') [13]. 20 ng of genomic DNA was used as the template for the PCR reaction in a 25-µl reaction mixture using a LA Taq (TAKARA BIO INC., Japan). The thermal cycle was carried out by first activating the Taq polymerase for 1 minute at 94˚C, followed by 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, and 5 minutes at 72˚C, and concluding with a 10-minute step at 72˚C for final extension. A 1 kb DNA ladder was used as a size indicator while electrophoresizing amplified PCR products on a 1.5% agarose gel in 1 × TAE buffer for 1 hour at 100 V and then stained while agitated in an EtBr solution (0.5% µg/mL). A UV transilluminator (Kodak Image Station 4000R; Molecular imaging system, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608) was used to see and photograph the stained gels. The amplification products were purified using the Maxwell® 16 DNA Purification Kits (Promega, USA). First BASE Laboratories Sdn Bhd in Malaysia conducted a bidirectional sequencing analysis on the purified PCR products.

MEGA6 and Bio-Edit were used to verify DNA sequences. Sequencing data were submitted to the NCBI, under accession number JUF0054. The closest matched taxa were found using a BLAST search with the ITS sequences. MEGA6 was used to perform multiple sequence alignments. Clustal W was used to convert the data from Fasta to MEGA format. The Akaike information criterion (AIC) was used to identify the evolution models. The Tamura-3 parameter model was chosen for study. The robustness of the branches was assessed using 1000 bootstrap repetitions and a max-trees setting of 1000 after performing maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) analyses [14].

2.4. Culture Media, Temperature and pH of *Cunninghamella blakesleeana*

Richard agar (RA), honey peptone agar (HPA), honey agar (HA), potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), and honey agar (HA) were used to test the mycelial development of the chosen fungus [15]. The medium will be adjusted to pH 6.5 before autoclaving. The pathogen's mycelial development on PDA was maintained at various temperatures (15˚C, 20˚C, 25˚C, 30˚C and 35˚C) in an incubator. Seven days after inoculation (dpi) was the time at which mycelial growth was observed [16]. On the PDA medium, the effect of pH on the mycelial growth of the pathogen was evaluated. The pH values employed were 5.0, 6.0, 7.0, 8.0, and 9.0 [17]. The medium was incubated at 30˚C for 10 days with 1 N NaOH or HCl before being autoclaved to a pH of 5, 6, 7, 8, and 9. Three different directions were used to measure the mycelial radial growth on each Petri dish.

2.5. Statistical Analysis

Standard statistical analysis tools, such as MS Excel, SPSS 16.0, MEGA 11.0 program, and BLAST tool, were used to analyze the data produced during the research

work.

3. Results and Discussion

3.1. Dry Fish Fungus of *Corica soborna*

Dried fish fungi, *Cunnighamella blakesleeana* was isolated from the selected SIS fish of *Corica soborna*.

3.2. Morphological Characteristics of *Cunninghamella blakesleeana*

Colonies at 25˚C grow rapidly, at first whitish, on aging becoming Cartridge Buff to Light Buff, mycelium was hyaline, often with granular content, hyphae and rhizoids present; conidiophores erected, with verticillate or solitary branches; vesicles subglobose to clavate, conidia globose to subglobose, smooth, pale-brown. zygospores globose, slightly compressed between the suspensors brownish, tuberculate; suspensors equal, smooth, hyaline heterothallic (**Figure 2**).

3.3. Molecular Characteristics of *Cunninghamella blakesleeana*

ITS4 and ITS5 primers were used to amplify the 740 bp long ITS region, which was then sequenced (**Figure 3**). Recent molecular phylogenetic investigations have shown that fungi at lower taxonomic levels may be identified extremely successfully using the internal transcribed spacer (ITS) region of genomic DNA. The internal transcribed spacer of rDNA is regarded as a region that varies between species and even within strains [18].

Based on the nucleotide sequences of the ITS regions of fifty-seven fungal species that were retrieved from the NCBI database for phylogenic analysis, the phylogenetic tree was formed. The percentage of homology between the rDNA sequence of the ITS region (JUF0052) and the previously discovered *C. blakesleeana* fungus with accession numbers MG569611.1, JQ683235.1, JN989285.1, JQ683237.1, and EU082781.1 was compared. There were eleven distinct clades discovered in the phylogenetic tree according to the highest parsimony method

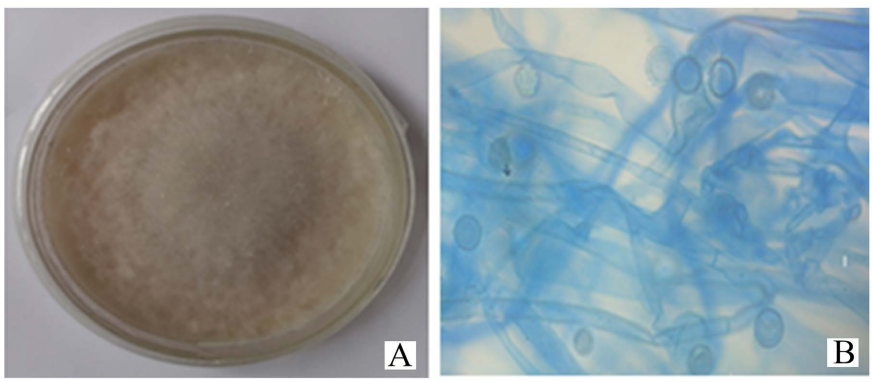

Figure 2. Morphological characteristics of *C. blakesleeana*. (A) mycelial growth of *C. blakesleeana* on PDA medium; (B) Microscopic view of mycelium and spore of *C. blakesleeana* (40× 10×).

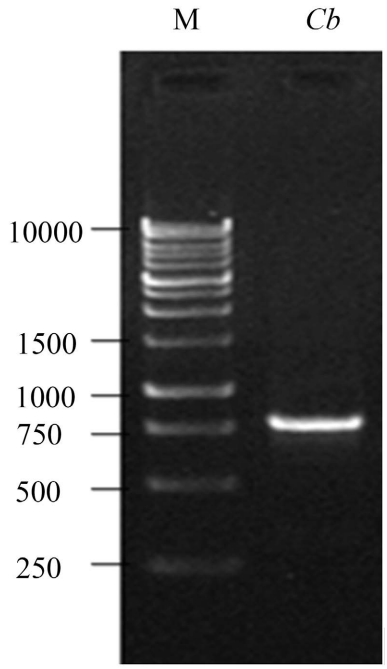

Figure 3. PCR products of the ITS region of isolated fungi. M, molecular size marker (1 kb DNA ladder); *Cb*. *Cunninghamella blakesleeana.*

(**Figure 4**). The ITS region sequences showed 98% to 100% reciprocal homologies. For our group's comparative investigations on the evolutionary connection with the chosen strain of *C. blakesleeana* (JUF0052), we used the sequencing data of the chosen NCBI GenBank strain (JN206290.1 *Heltermyces radiatus*) as outgroup. The findings showed that a single cluster contains every distinct species of *C. blakesleeana*. According to Alam *et al.* [19], ITS sequences were genetically stable or exhibit minimal change within the species, but they differ across species within a genus. It was evident from the molecular data that the fungus we had studied was *C. blakesleeana*.

3.4. Effect of Culture Media, Temperature and pH of *Cunninghamella blakesleeana*

Figure 5 shows the effect of several culture media, including PDA, CA, RA, PSA, HPA, and HA, on the mycelial growth of *C. blakesleeana*. The findings indicated that the PDA medium had the maximum mycelial growth of *C. blakesleeana* (95.33 mm), followed by the PSA medium, while the HPA medium had the lowest growth (65.50 mm). De-Hoog *et al.* [20] found that Richard's broth and Sabouraud's broth among the liquid media tested appeared to be better than other media for the growth of tomato early blight-causing fungi *Alternaria solani*. These findings support the current findings of *C. blakesleeana*. On PDA, *Alternaria brassicae* showed the most significant growth, as was seen. The researchers that studied *Alternaria solani*, Kumar *et al.* [21], also reported obtaining the same investigation. Under *in vitro* conditions, the PDA medium's growth pattern was the best.

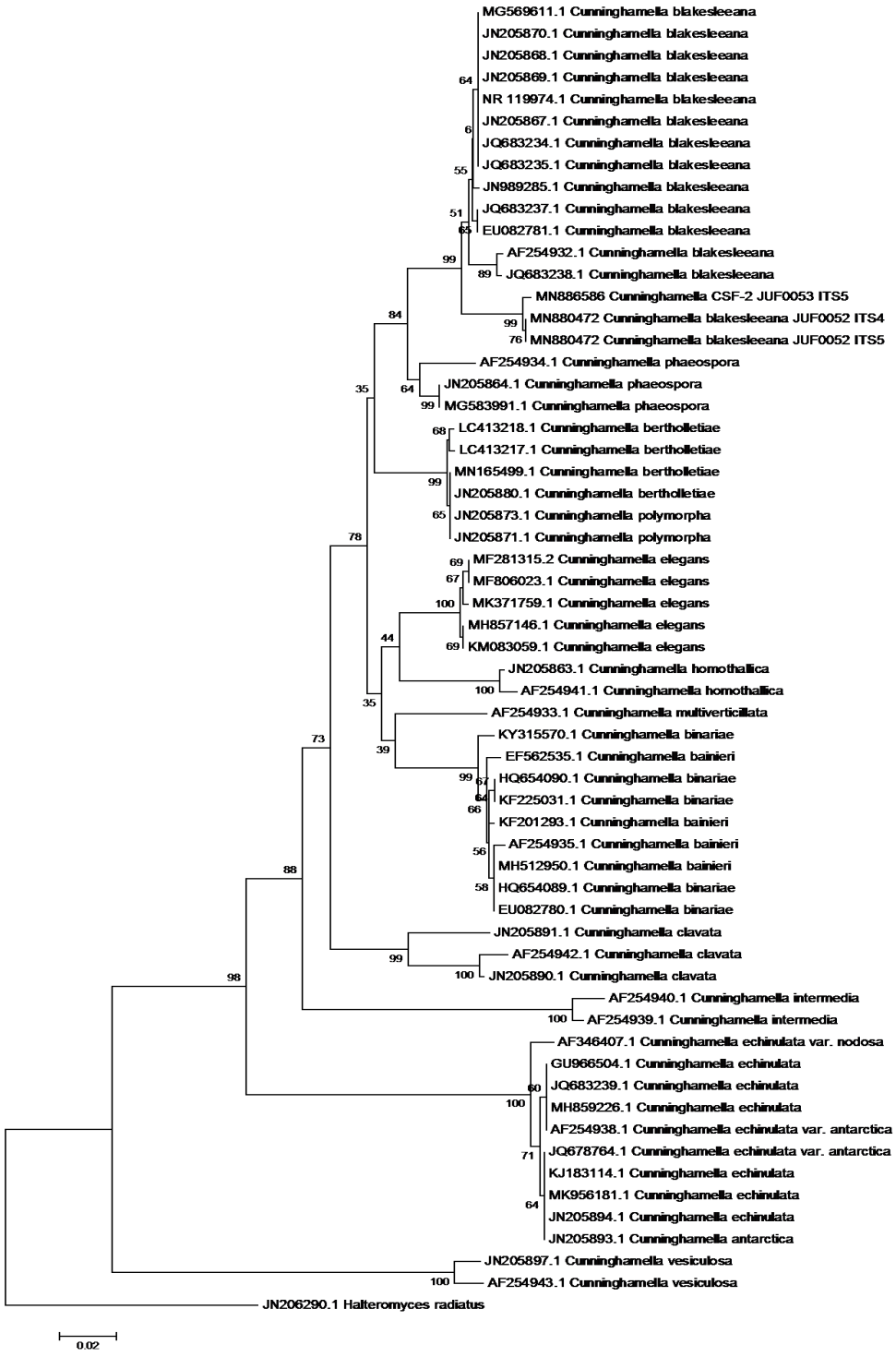

Figure 4. The maximum likelihood tree was derived using the bootstrap value of the ITS sequencing dataset of the investigated organism. Our organism is marked with JUF0052.

The findings of the current investigation, which examined the impact of temperature on *C. blakesleeana* radial mycelial growth on PDA medium incubated at five different temperatures: 15˚C, 20˚C, 25˚C, 30˚C, and 35˚C are displayed in **Figure 6**. According to the findings, *C. blakesleeana* grew at a rate that was

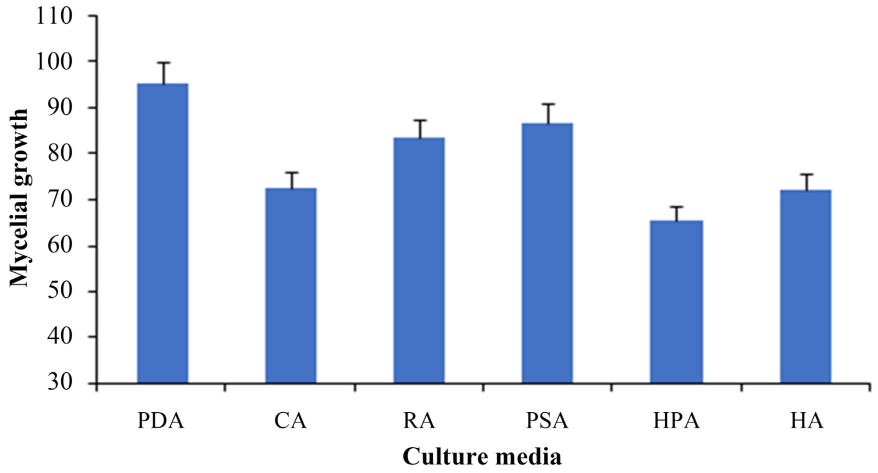

Figure 5. Effect of various culture media on the *Cunninghamella blakesleeana* mycelial growth at 7 dpi. CA: carrot agar; PSA: potato sucrose agar; RA: Richard agar; HPA: honey peptone agar; and HA: honey agar.

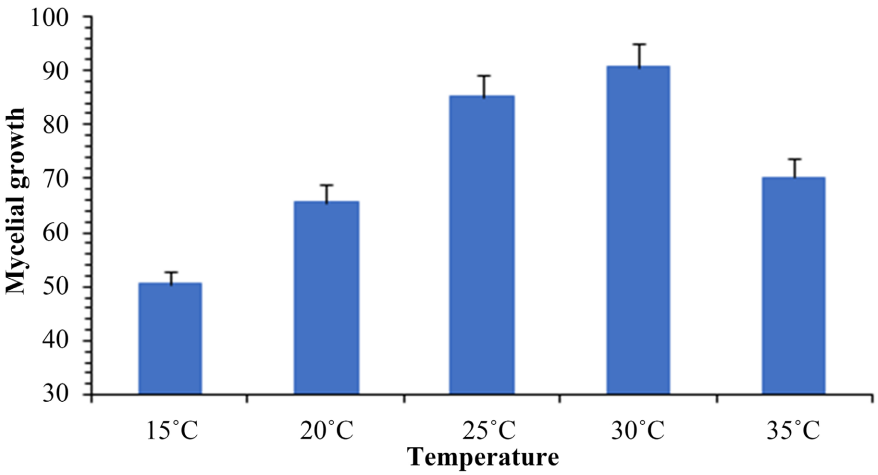

Figure 6. Effect of different temperatures on mycelial growth of *C. blakesleeana*.

maximum at 30 degrees Celsius and then at 25 degrees Celsius. The highest levels of mycelia growth and sporulation of *C. blakesleeana* were recorded in our experiment at 30˚C, which is similar to previous findings of Iwen *et al.* [22] who noted a significant decrease in mycelial growth and sporulation between 30˚C and 35˚C. The findings also show that *C. blakesleeana* mycelial growth is unaffected by temperature, and that the right mix of these factors may be used to prevent or delay the growth of the mold in order to minimize product losses and the economic impacts of fungal contamination.

Although pH is an important variable in comprehending the ecology of spoilage fungus, the experimental plates in this study were incubated at five different pH values, namely 5, 6, 7, 8, and 9. *C. blakesleeana* showed maximum mycelial growth (88.25 mm) at pH 7, followed by pH 8 and pH 6, and minimal mycelial growth at pH 9 (**Figure 7**). These findings conflict with prior studies by Sonyal *et al.* [23], who found that *Ceratocystis fimbriata* grew most effectively at pH 7.5,

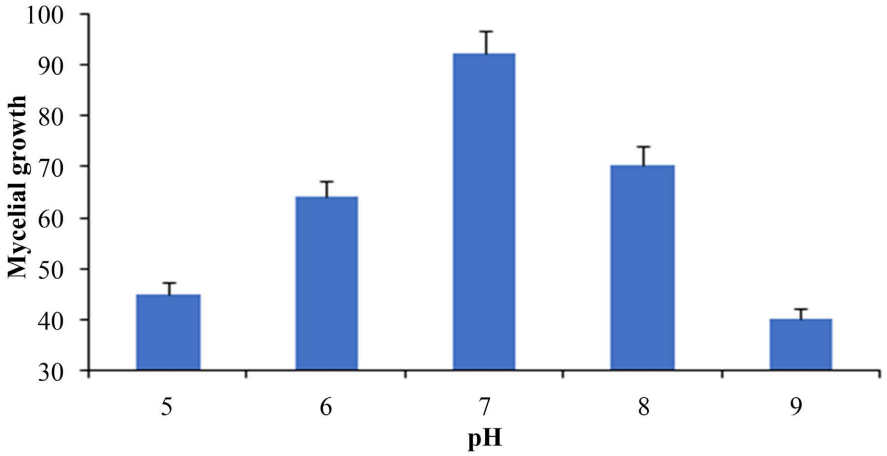

Figure 7. Effect of different pH on the mycelial growth *C. blakesleeana*.

followed by pH 7.0 and pH 8.0. From pH 2.0 to pH 5.5 and above pH 9.0, the fungus growth slowed down. The highest mycelial growth of *Ceratocystis paradoxa* was observed by Yadahalli *et al.* [24] when the pH of the medium was between 6.0 and 7.5. As a result, the findings showed that *C. blakesleeana* grew well in neutral conditions.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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