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Mycoremediation of reactive red HE7B dye by Aspergillus salinarus isolated from textile effluents

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ABSTRACT

Reactive dyes are widely utilized in the textile industry due to their advantageous properties of vivid color, water-fastness, and simple application procedures with minimal energy usage. The toxicity of most azo dyes is a significant environmental concern, as effluents from dye processing and manufacturing sectors are known to be carcinogenic and mutagenic to numerous species. These issues are more grievous in Bangladesh, one of the largest exporters of apparel. This study aimed to isolate and identify potential fungal strains from textile effluent that are capable of degrading Reactive Red HE7B dye (a sulphonated reactive azo dye), a widely used dye in local thread dyeing industries. Dye degradation assay was performed in potato dextrose broth supplemented with 50 mg/l Reactive Red HE7B and the degradation rate was measured by a UV spectrophotometer. DNA extraction, quantification, PCR, internal transcribed spacer (ITS) sequencing, and phylogenetic analysis were performed to identify the selected fungi. Among the isolates, the three best performing strains TEF -3, TEF -4, and TEF -5 showed 97.41%, 93.12%, and 82.89% dye degrading efficacy after 96 h of incubation, respectively. All three strains, TEF-3, TEF-4, and TEF-5 showed similarity with *Aspergillus salinarus* (accession no. NR_157473.1) and the similarity percentages were 97.02, 96.95, and 95.28 respectively. Interestingly, this study probably the very first indication of textile dye degradation by *Aspergillus salinarus* strains. Thus, these fungal strains possess the prospectiveness to be utilized in the textile wastewater treatment plants, since the isolates demonstrated the substantial capacity (>80%) to degrade Reactive Red dye after 96 h of incubation.

1. Introduction

Water pollution from textile industries is a worldwide environmental crisis, especially in the leading exporter countries of fabrics. It is estimated that annually 700,000 tons of dyes are produced worldwide and 280,000 tons/year are released to the environment mainly in the form of effluents (Berradi et al., 2019). These colorants pose a vital threat to the aquatic and human life systems. For instance, these dyes are generally no degradable and impede the access of sunlight to water bodies, thereby obstruct the photosynthesis process in the aquatic ecosystem. Moreover, they pose problems for fishes and water-dwelling algae (Gita et al., 2017). Besides, such dyes are reported to be carcinogenic and also cause breathing and allergic problems (Lima et al., 2007, Lellis et al., 2019;.

Over the past several decades, physical and chemical methods for the decolorization of textile wastewater have been employed. Unfortunately, these approaches resulted in the production of undesirable by-products, higher sludge formation, higher costing, indiscriminate usage of chemicals (Sghaier et al., 2019). For instance, chemical techniques are unsustainable and produce hazardous sludge, whereas physical dye degradation processes are time-consuming, expensive, and take up a large space (Routoula and Patwardhan 2020). Hence, there is an ultimate urgency for a more natural, environmentally efficient, and cost-effective alternative for the remediation of these widely used dyes. Bioremediation is an eco-efficient, cost-effective and publicly acceptable approach compared to physicochemical remedial methods of pollutions (Azubuike et al., 2016, Banat 1996, McMullan 2001). In particular, bacteria and fungi are two extensively studied candidates for

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bioremediation (Gouma et al., 2014).

Usage of microorganisms for the degradation of azo dyes has been initiated in the 1970s. Horitsu *et al.,* (1977) first reported the degradation of p-Aminoazobenzene (PAAB) by *Bacillus subtilis* (Horitsu et al., 1977). Further, *Aeromonas hydrophila* var. 24B had been reported to degrade azo dyes in 1978 (Idaka et al., 1978), followed by *Bacillus cereus* (Wuhrmann et al., 1980). Extensive number of bacteria for textile dye degradation have been identified and characterized over the past several decades. For instance, *Enterobacter* sp. CV–S1 degraded 150 mg/l Crystal Violet dye after 72 h of incubation at pH 6.5 and temperature 35°C (Roy et al., 2018). Moreover, *Enterobacter* sp. CM-S1 degraded Malachite Green dye at a concentration of 15 mg/l after 144 h of incubation (Chandra et al., 2020). Moreover, *Klebsiella variicola, Enterococcus faecalis, Bacillus* sp. strain CH12, *Bacillus subtilis* ATCC6633, *Bacillus subtilis* ISW1214 have been reported to degrade different Reactive Red dyes (Chengalroyen and Dabbs 2013; Shindhal et al., 2021).

The white-rot fungi are by far the most studied dye-decolorizing microorganisms (McMullan et al., 2001). Fungi produce extracellular ligninolytic enzymes including laccase, manganese peroxidase, and lignin peroxidase, which are outstanding degraders of organic molecules like textile dyes in an eco-friendlier manner (Sen et al., 2016) The first ligninolytic peroxidases were called lignin peroxidase (LiP) (Glenn and Gold 1983) and manganese peroxidase (MnP) (Kuwahara et al., 1984), which were isolated from *Phanerochaete chrysosporium*. Notably, *P. chrysosporium* was the first white-rot fungus reported to biodegrade dyes (Chengalroyen and Dabbs 2013). Laccase-mediated enzymatic biodegradation and decolonization of textile effluents and colorants is a highly effective treatment method for industrial wastewater containing textile dyes (Shindhal et al., 2021). Fungal laccases are formed by almost all wood and litter transforming basidiomycetes as part of the ligninolytic enzyme system and are divided into three groups based on their basic properties (Call and Mücke 1997).Various fungi have been reported to be a significant decontaminant of different textile dyes. *Phanerochaete chrysosporium* was the first discovered white-rot fungi that were capable of degrading dyes (Glenn and Gold 1983). Besides, *Aspergillus sojae, Myrothecum verrucaria, Neurospora crassa, Pycnoporus cinnabarinus, Trichoderma* sp., *Candida* sp. had been reported to decolor dyes over the past few decades (Banat et al., 1996). Moreover, *Aspergillus niger* degraded 81.85% basic fuchsin dye at 25 ° C (Rani et al., 2014). In addition, 100% degradation of Reactive Red dye by mixed fungal culture had been reported by Nascimento et al., (2011)

Reactive dyes are widely utilized in the textile industry due to their advantageous properties of vibrant colors, water-fastness, and simple application procedures with minimal energy usage (Bankole et al., 2017). The present research aimed at isolating and characterizing Reactive Red HE7B dye degrading fungi from the textile effluents for possible use in the industrial process of bioremediation.

2. Materials and Methods

2.1. Sample Collection

Untreated textile water for the isolation of dye-degrading fungi was collected from the drainage canal of Rana Textile Limited, Kumarkhali, Kushtia, Bangladesh. The sample was collected in a sterile plastic bottle and transferred to the laboratory immediately followed by storing at 4^0 C. The physical parameters of textile water, such as color, pH, and temperature were measured.

2.2. Dyes and Media

The Reactive Red HE7B dye was purchased from the local market near the textile industries of Kumarkhali, Kushtia, Bangladesh. The dye used in this experiment is commonly used by local textile manufacturers. For the isolation and maintenance of fungal strains and dye degradation, potato dextrose agar (PDA), potato dextrose broth (PDB), and nutrient agar (NA) were used, procured from (Sigma Biotech, USA).

2.3. Isolation of Fungi from Textile Effluents

Nutrient agar media was prepared for the primary isolation of potential fungal strains. The sample was then serially diluted and spread on nutrient agar. After incubating at 37°C for 48h, six fungal colonies were selected. Potato dextrose agar was prepared for the pure culture of the selected fungal strains. Further, Selected colonies were sub-cultured on PDA and incubated at 30°C for 96 h. For obtaining the pure culture of fungal strains, repeated sub-culturing was performed by cutting the edge of growing fungi from PDA and re-inoculating on new PDA plates.

2.4. Liquid State Dye Degradation by Isolated Fungi

Dye degradation at the liquid state was performed in the PDB. Briefly, Reactive Red HE7B dye (at a concentration of 50 mg/l) was mixed separately into a test tube containing 10 ml of PDB. One loop full of each isolated strain was inoculated separately into the test tubes containing PDB supplemented with respective dyes. Thereby, each isolated strain was examined for their capability of degrading Reactive Red HE7B dyes. Un-inoculated PDB supplemented with dye was considered as a negative control. All the test tubes were incubated at 30°C and observed daily for dye degradation and every experiment was triplicated.

2.5. Dye Degradation Assay

Dye degradation assay at liquid medium was performed according to Roy et al., (2018) by a photoelectric colorimeter (Roy et al., 2018). The percentage of dye degradation by each fungal strain was examined by analyzing the depletion of absorbance at absorbance maxima (λ max). Un-inoculated PDB containing appropriate dyes was used as a reference. Concisely, 2 ml of each decoloured sample was transferred to an Eppendorf tube and centrifuged at 10000 rpm for 10 minutes to separate the biomass. After that, the supernatant was further transferred to the cuvette and the percentage of degrading Reactive Red HE7B dye by fungi was determined by absorbance at 545 nm according to the following formula (Chandra et al., 2020). Each experiment was replicated three times. Further, the highest Reactive Red HE7B dye degrading fungi were selected for further characterizations.

$$DyeDegradation(\%) = \frac{InitialOD - -FinalOD}{InitialOD} \times 100$$

2.6. DNA Extraction of the Best Performing Fungal Strains

DNA of the highest Reactive Red HE7B dye degrading fungi were extracted following the procedure of Chi *et al.,* (2009) with some modifications (Chi et al., 2009). Concisely, fungal strains were inoculated on an agar medium and fungal mass was transferred to an Eppendorf tube containing extraction buffer. Further, the reaction mixture was mechanically ground and centrifuged (5000 rpm for 10 minutes) to remove the cell debris. The supernatant was further transferred to another Eppendorf tube containing 0.3 ml 2-propanol. The reaction mixture was again centrifuged at 12,000 rpm for 10 min and the supernatant was discarded. Finally, the DNA pellet was dissolved by distilled water and vortexing.

2.7. PCR Amplification

Polymerase chain reaction (PCR) was performed by thermal cycler, using ITS4 Primer: 5'TCC TCC GCT TAT TGA TAT GC3' and ITS5 Primer: 5'GGA AGT AAA AGT CGT AAC AAG G3'. A total of 25 μl reaction mixture for PCR contained Master Mix (12.5 μl) (dNTPs, Buffer, MgCl2, Taq Pol), template DNA (1 μl; 65 ng/μl), both primer (1μl each; 20

pMol), ddH_2O (9.5 μl). The polymerase chain reaction was performed by Gene Atlas, Model: G2, Origin: Astec, Japan. The following steps were: preheating the reaction mixture at 95°C for 5 minutes, followed by 35 cycles of 30 seconds of denaturing at 95°C, 30 seconds of annealing at 48°C and 60 seconds of elongation at 72°C with a final extension at 72°C for 5 minutes.

2.8. ITS Sequencing

ITS sequencing of the purified PCR product was further conducted by DNA sequences in Invent Technologies Ltd, Dhaka. In the next step, the nucleotide sequences generated from the ITS sequencing were analyzed through ChromasPro (Version 2.6.6).

2.9. Similarity test and phylogenetic analysis

The BLAST was performed in order to evaluate the similarity between isolated fungi strains with known sequences previously deposed in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). More specifically, internal transcribed spacer regions from fungal type and reference material were selected from the rRNA/ITS dataset (for similarity test). The neighbor-joining method was used to determine an evolutionary relationship through the online software of NCBI, where the neighbor-joining tree method, Taxonomic name in a sequence level, and 0.75 maximum sequence difference were selected to generate a phylogenetic tree.

3. Results and Discussion

3.1. Physical Characteristics of the Textile Effluent

In general, textile effluents carry a notable amount of unutilized dyes which causes unusual coloration (Errais et al., 2010). The color of the effluent was black. In addition, the pH and temperature of the effluent are important parameters as they influence the treatment process and various chemical and biological processes in the water (Chandra et al., 2020). The pH of the effluents was slightly acidic (6.4-6.6) and the temperature was 24°C.

3.2. Isolation of dye degrading fungi and reactive red dye degradation

Six fungal strains were isolated from the textile effluents. They were designated as TEF-1, TEF-2, TEF-3, TEF-4, TEF-5, and TEF-6. These fungal strains were maintained on PDA and stored at 4 °C. In dye degradation assay, these six fungal strains showed gradual increasing of Reactive Red HE7B dye degradation up to the concentration of 50mg/l after 96 h incubation (Table 1). However, degradation capabilities of all six strains were dropped when more than 50mg/l dye concentration was used in the assay. Strain TEF-1 showed the lowest degradation (35.51%), whereas TEF-3 exhibited the highest degradation (97.41%). Moreover, TEF-4 and TEF-5 also demonstrated remarkable dye degradation, 93.12% and 82.89% respectively. Results are shown as mean ± standard deviation. The dye degradation curve (Fig. 1) shows the Reactive Red HE7B degradation rate by six fungal strains after different time intervals.

Table 1
Degradation Rates of Reactive Red HE7B Dye by Six Fungal Strains.

Fungal Strain	IOD	FOD	Degradation (%)
TEF-1	0.32±0	0.206±0.005	35.51
TEF-2	0.32±0	0.111±0.003	65.13
TEF-3	0.31±0	0.008±0.001	97.41
TEF-4	0.32±0	0.022±0.002	93.12
TEF-5	0.31±0	0.053±0.002	82.89
TEF-6	0.32±0	0.129±0.004	59.47

IOD: Initial optical density; FOD: Final optical density

3.3. Molecular identification of higher dye degrading fungi

Three fungal strains (TEF-3, TEF-4, and TEF-5) with higher dye degrading capability were identified by ITS sequencing (Supplementary Table 1 and Supplementary Table 2). Gel electrophoresis of the PCR product showed a band around 550-650 base pairs (Supplementary Fig. 1). BLAST exploration of the nucleotide sequences derived from ITS sequencing exhibited homology with the known sequences in the NCBI gene bank. Isolated fungal strains TEF-3, TEF-4, and TEF-5 demonstrated 97.02%, 96.95% and 95.28% similarity with *Aspergillus salinarus* CBS 138583, respectively.

3.4. Phylogenetic tree analysis

All three strains showed similarity with the strains *Aspergillus salinarus* (NR_157473.1). Several species of *Aspergillus* genus have been identified with textile dye degrading capacity, for instance, *Aspergillus niger* degraded 97% Congo Red dye after six days of incubation (Asses et al., 2018). In addition, *Aspergillus flavus* was reported to utilize Malachite Green dye as a sole carbon source and showed 99.78% degradation efficacy (Ali et al., 2009). Chemical structures of these dyes degraded by *Aspergillus* species are illustrated in Fig. 2. In the phylogenetic tree, all three strains appeared in the same cluster of *Aspergillus salinarus* (NR_157473.1) (Fig. 3). Therefore, this finding indicated that all the three isolated strains having Reactive dye degrading capacity were the strains of *Aspergillus salinarus*. However, to the best of our knowledge, this is the first time an *Aspergillus salinarus* strain has been recorded as a biological agent of textile dye degradation.

In a study, Ibrahim et al., (2017) reported that *Aspergillus* sp. and *Pleurotus* sp. at pH 5 exhibited 63% and 60% degradation of Reactive Red- 120 dye, respectively. Ibrahim et al., (2017) used Reactive Red-120 dye at a concentration of 10 mg/l and 20 mg/l in their study, whereas in current study, two potential fungal strains were able to degrade >90% of the Reactive Red HE7B dye at a much higher concentration (50 mg/l). However, mixed fungal culture revealed significant potency in degrading the Reactive Red dye. For instance, mixed fungal culture was able to degrade 100% Reactive Red 198 dye (100 mg/l) through degradation and adsorption mechanisms (Nascimento et al., 2011). Though, the incubation period was seven days in that particular study which was almost double the present study (96 h). In addition, *Aspergillus bombycis* showed 99.02% degradation of Reactive Red 31 dye (20 mg/l) after 12 h of incubation (Khan and Fulekar 2017). The degradation rate in the Khan *et al.,* (2017) study is almost similar to the present study, however, dye concentration and incubation time were lower than the present study (Khan and Fulekar 2017).

4. Conclusion

Although textile effluent degradation or decolorization is a difficult process, several notable microorganisms including bacteria and fungi have been identified as a potential degrader of textile dye. In the present study, three strains of *Aspergillus salinarus* have been isolated from the textile effluent with the capacity of degrading Reactive Red HE7B dye. Notably, *Aspergillus salinarus* was able to degrade up to 97.41% Reactive Red HE7B dye at a concentration of 50 mg/l after 96 h of incubation at 30°C. This study reveals the remarkable potency of *Aspergillus salinarus* for usage in wastewater treatment plants for the degradation or decolorization of Reactive Red dye. Furthermore, to the best of our knowledge, this study indicates the first reporting of *Aspergillus salinarus* strains as a biological agent of textile dye degradation.

Author contribution

MMS (Researcher), SKB (Associate Professor) and MMR (Professor) contributed to design the research plan and did the study. MMS (researcher) KRA (Researcher), SBS (Researcher), MSH (Researcher),

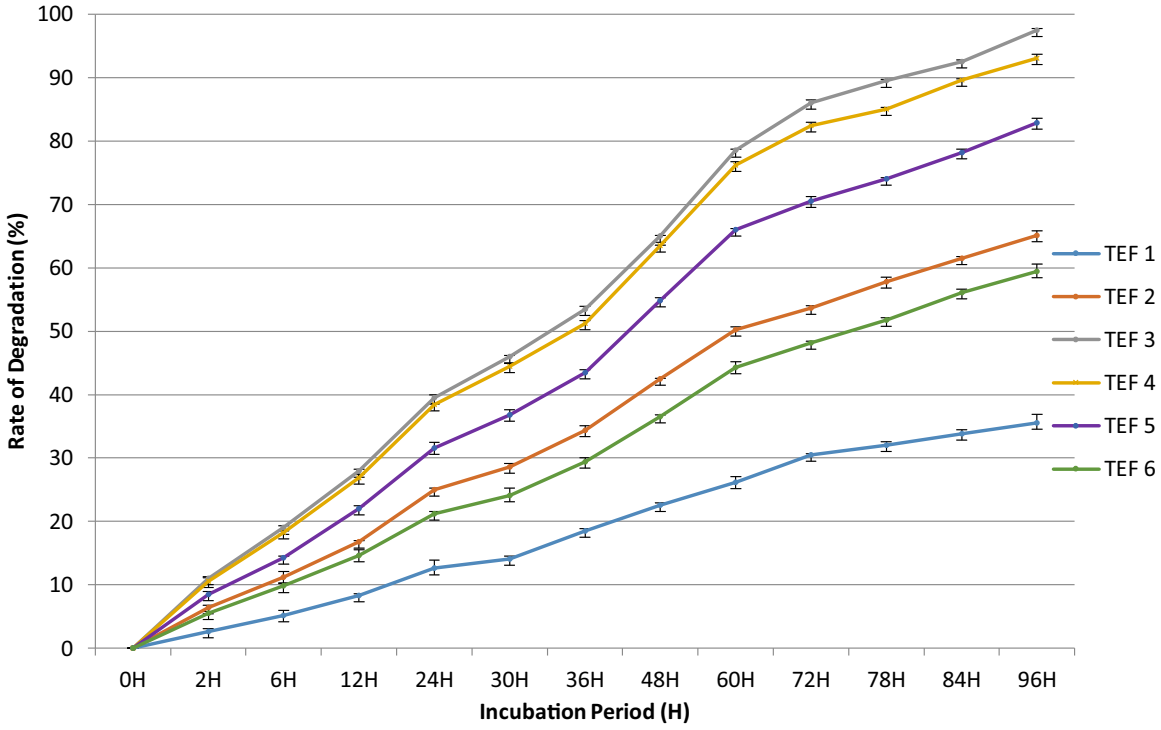

Fig. 1. Dye degradation curve by six fungal strains. Notably, after 24 h of incubation, 39.5%, 38.4%, and 31.6% degradation were observed by TEF-3, TEF-4, and TEF-5 respectively. Finally, after 96 h of the incubation period, these three isolates demonstrated 97.4%, 93.1%, and 82.89% of Reactive Red HE7B dye degradation respectively. In addition, TEF-1, TEF-2 and TEF-6 exhibited 35.51%, 65.13% and 59.47% Reactive Red HE7B dye degradation respectively after the incubation period.

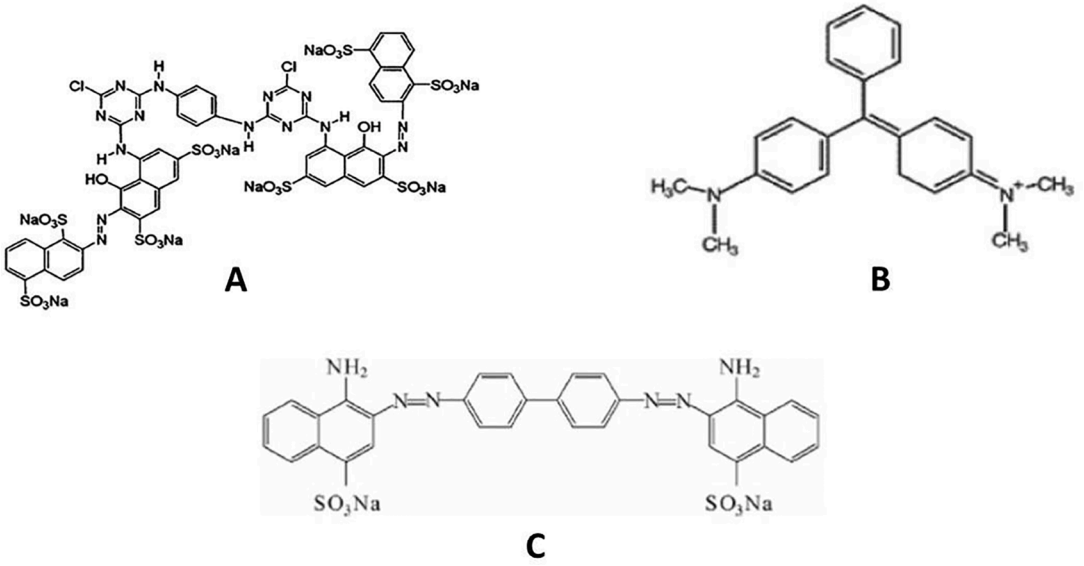

Fig. 2. Structure of Congo Red, Malachite Green and Reactive Red HE7B dyes: (A) represents Reactive Red HE7B; (B) represents Malachite Green; (C) represents Congo Red (Bankole et al., 2017; Chaturvedi and Verma 2015; Yokoyama et al., 2010).

MSAK (Researcher) FTZ (Researcher) and MRH (Researcher) performed lab work and collected the data. MMS (Researcher), SKB (Associate Professor), MMR (Professor) contributed to data analysis and interpretation. MMS (Researcher), SKB (Associate Professor), SBS (Researcher), and MRH (Researcher) drafted the article with the help of all authors. SKB (Associate Professor) and MMR (Professor) reviewed the manuscript critically. SKB (Associate Professor) supervised the project. All authors read and approved the final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Supplementary materials

Supplementary material associated with this article can be found, in

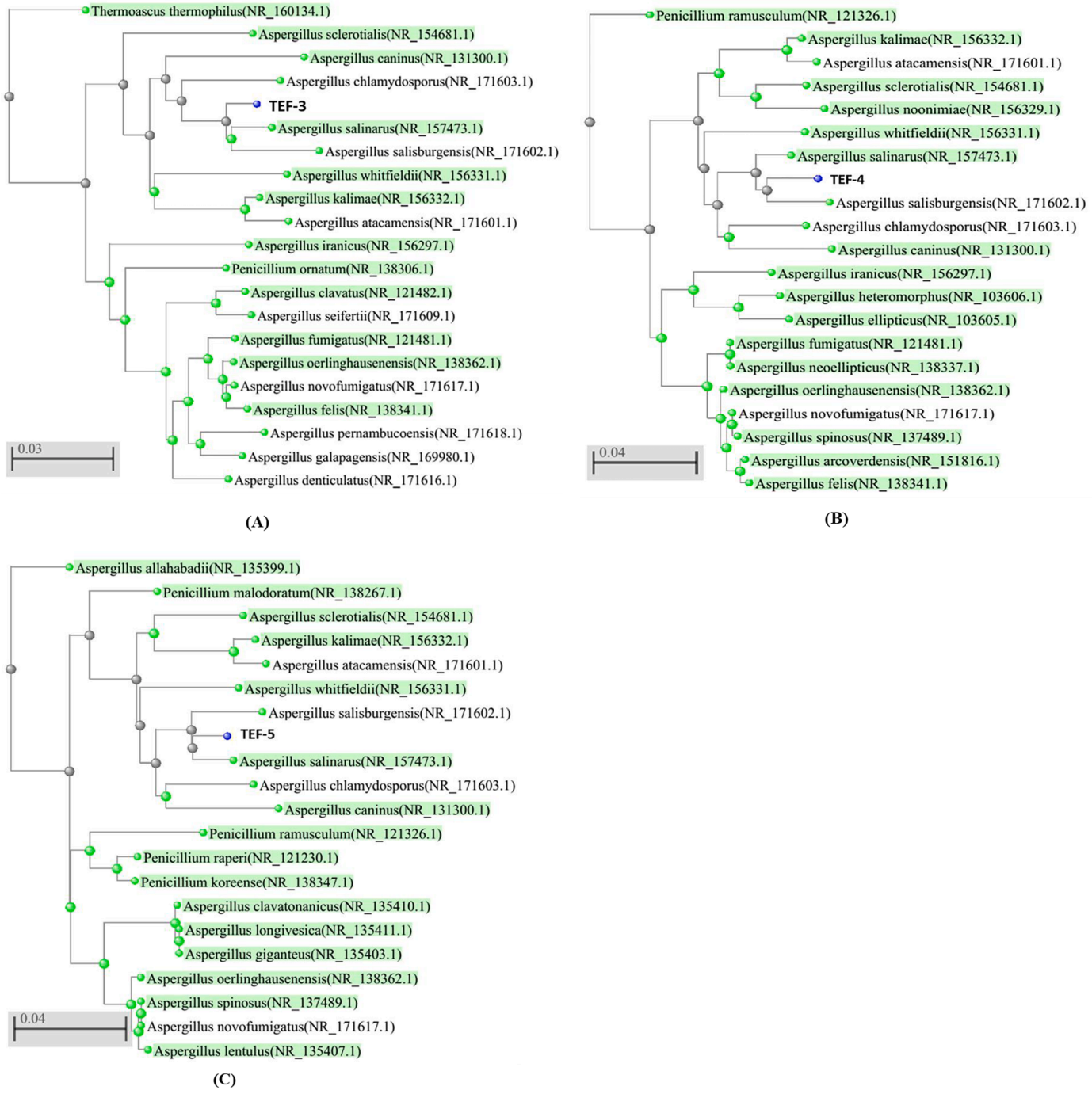

Fig. 3. Phylogenetic tree of three fungal strains. A, TEF-3; B, TEF-4; C, TEF-5 showing similarity with the same Aspergillus salinarus (NR_157473.1) strain.

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