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Molecular identification of fungi isolated from used rubber and estimates of their ability to degrade rubber

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Abstract

This study explores the use of fungi for sustainable waste tire disposal. Waste tires pose significant environmental and health risks due to slow degradation and harmful disposal methods. Isolated fungi were identified morphologically and using molecular techniques. The internal spacer of the 16S rRNA gene sequencing technique identified genetic diversity among the fungal isolates. The findings suggest that fungi, especially *Aspergillus nidulans*, offer a viable and eco-friendly solution for waste tire management. The ITS1/ITS4 gene was used to identify *Aspergillus nidulans*, *A. fumigatus*, and *A. oryzae*. Four *Aspergillus* (*A. niger, A. nidulans, A. fumigatus, and A. oryzae*) isolates were evaluated for their ability to degrade rubber. *Aspergillus nidulans* recorded the highest growth and enzymatic activity, including laccase and peroxidase production. Scanning Electron Microscope (SEM) analysis revealed significant rubber degradation by *A. nidulans*. This study emphasizes the potential of fungal species in the sustainable decomposition of rubber, providing an environmentally acceptable alternative for tire waste management.

Keywords: Rubber degradation; *Aspergillus*; ITS 16SrDNA; Ecofriendly degradation.

Introduction

Natural rubber (NR) has been essential for nearly a century, widely used in tires and other products, with global consumption estimated at 12.5 million metric tons in 2013 [1]. Despite the development of synthetic rubber (IR), which shares a similar chemical structure with NR, both types are biodegradable and remain crucial in over 40,000 products [2]. However, discarded tires pose significant environmental challenges due to their slow degradation and difficulty in recycling, contributing to pollution and waste [3]. Efforts to address this issue focus on recycling and reclaiming rubber through mechanical, chemical, and microbial methods [4].

Microorganisms, especially fungi like *Aspergillus* species, have shown promise in degrading complex polymers like rubber [5]. *Aspergillus niger*, for instance, produces enzymes like cutinase and rubber oxygenases that can break down rubber's molecular structure [6]. These organisms offer a biotechnological solution for recycling rubber, as their metabolic pathways allow for the breakdown of tough compounds under environmental stress [7]. Additionally, enzymes like laccases, found in fungi, aid in biodegradation and have broad industrial applications in bioremediation [8], [9].

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The ITS region is the major marker for fungal species identification because of its diversity and capacity to discriminate between closely related species. It has been utilized successfully in clinical settings for accurate fungal identification, as evidenced by our search with 89.2% species-level concordance in clinical isolates [10]. ITS sequencing is also useful in environmental research, such as determining fungus diversity in soil and dairy products. The ITS3/ITS4 primer combination has been proven to outperform other primers in soil fungal biodiversity studies [11], and ITS sequencing indicated high fungal variety in dairy products [12] to identify the isolated fungus at the species level, the recovered fungal DNA was amplified by PCR with a particular internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared to comparable sequences in GenBank (NCBI) [13].

The aim of this work is to study the molecular and enzymatic characteristics of rubber-associated fungi and their role as ecofriendly rubber degradation

Materials and Methods

1. Samples Collection

Samples of used tires and surrounding soil (Fig. 1) were collected from Al-Shallal area in Aswan city. Sampling sites were carefully selected to ensure a good representation of the local environment. Samples were collected under clean conditions to avoid contamination and placed in sterile plastic bags, then transported to the laboratory.

Fig. 1 Used tires sample in Al-Shallal area in Aswan city.

Isolation and identification of fungi:

One ml of an aqueous suspension of soil or deteriorated rubber was mixed with 0.5 gm of powdered natural rubber and 5 ml of Sabouraud culture medium. The mixture was then poured into a sterilized Petri-dish containing 20 ml of the same medium. Cultures were incubated at 28°C for 15 days [14]**.** Isolated fungi were identified morphologically and by DNA sequencing.

Fungal DNA extraction methods

DNA extracted from fungal by using a multi-step process including growing the fungus in liquid, lyophilizing the fungal samples, disrupting cell walls, removing proteins with phenol and chloroform, and precipitating DNA with ethanol or isopropanol.

DNA extraction

Fungal DNA isolates were extracted using the CTAB procedure [15] [16], with some modifications. Fungal isolates were grown in LB (Luria Bertani, Miller) [17], which is composed of 10 g of peptone, 5 g of yeast extract, and 3 g of NaCl to prepare 1 L of liquid media for 5-7 days in

a shaking incubator at 28ºC. The fungal mycelium was filtrated using filter paper, and then 1 ml of extraction buffer cetyltrimethylammonium bromide (CTAB buffer) was added, which contains (Tris-HCL 1.576 g, NaCl 8.18 g, EDTA 0.744 g, 2% CTAB). H2O at pH 8.4) was added 50 µL of β-mercapto-ethanol to the fungal mycelium, pounded it with a mortar and pestle. Incubate at 65ºC for 20 minutes before adding an equivalent amount of chloroform/isoamyl alcohol and vortexing. Spin for 10 minutes at 10,000 rpm in a bench centrifuge, then transfer the aqueous phase to a new Eppendorf tube. To combine the materials, fill the remaining volume of the Eppendorf tube with 100% ethyl alcohol or isopropanol, seal it, and invert it several times. Using a bench centrifuge set at 10,000 rpm for five minutes, Exclude the supernatant. After carefully discarding the supernatant, add approximately 1 milliliter of cool 70% (v/v) ethanol. Centrifuge the mixture for five minutes at 10,000 rpm on a bench centrifuge to extract the DNA. After discarding the supernatant and allowing the remaining ethanol to evaporate for a day, elute the DNA pellet in 50 µL of water. The internal transcribed spacer (ITS) was used for fungus identification.

Electrophoresis on Agarose Gel

The DNA fragments were resolved using ultra-pure agar (1), and the agarose gel was prepared using TBE buffer, and the run was carried out for 30 minutes at 90 volts. Gel band were identified by applying a UV transilluminator.

Conditions for ITS isolation and PCR amplification:

The ribosomal internal transcribed spacer was amplified by PCR using primers. ITS 1 (5̓-TCCGTAGGTGAACCTGCGG-3̓) and ITS 4 (5̓-TCCTCCGCTTATTGATATGC-3̓). In a thermal cycler, the reaction was first denatured for five minutes at 95°C. It then underwent thirty seconds of denaturation at 95°C, thirty seconds of primer annealing at 50°C, one minute of primer extension at 72°C, and five minutes of extension at 72°C.

Amplified PCR product gel electrophoresis:

1.5% agarose gel was used to separate the PCR product in 1X TBE buffer with 0.5µg/ml of ethidium bromide, maintained at a constant 80V for 30 minutes. As a size marker, a 1 kb DNA ladder was used. UV light was used to view the gel.

Genetic characterization using ITS gene sequencing

DNA sequencing of the ITS gene was used to identify specific fungal isolates. According to the Gene Analysis Unit (Macrogene Inc., Seuol, Korea), the sequencing was carried out as follows [18]: The isolated DNA served as a template for a PCR reaction that amplified the ITS gene. The primer sequences ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [19], which target the fungal internal transcribed spacer 1 (ITS1) region of the rRNA genes, were used to build amplicon libraries for DNA sequencing. Thermo Scientific Phusion DNA polymerase (0.4 U) was used in a 20-µL reaction for amplifying the fungal DNA (1 µL). Additional ingredients were 0.2 mM dNTPs, 3% dimethyl sulfoxide, 0.5 µM of each primer, and high-fidelity DNA polymerase buffer (HF). First, the reaction was denatured for 30 seconds at 98ºC. Next, it was extended for 10 minutes at 72ºC after 30 cycles of 10 seconds at 98ºC, 30 seconds at 55ºC, and 30 seconds at 72ºC.

Analysis of the sequencing data

To identify the isolated fungi, a BLAST search (NCBI) was performed on the gene sequence against full sequences present in the GenBank database. The phylogenetic tree was built using MEGA11 software, which combined sequences acquired from the GenBank database.

Estimates the Effect of incubation temperature (28˚C and 40˚C) on the fungal growth on media with and without rubber

Seven days fungal culture on Nette medium was used for spore suspension preparation. One ml of spore suspension was transferred to 1000 ml flask contain screening medium. The Flasks incubated at 28°C and 40°C with shaking (200 rpm) for 15 days. Then, the biomass was recovered by filtrate through dried filter paper. The fungal mycelia were dried at 60°c overnight for dry weight determination [14].

Screening the ability of the studied fungi to degrade rubber in Nette Medium broth

Preparation of rubber

Air-dried rubber parts (1cm) were weighed and sterilized in 70 % ethanol for 2 min, dried well and then kept under UV lamp for 30 min.

Preparation of fungal inoculum

Seven days fungal culture growing on Nette medium were used for spore suspension preparation, which prepared by taken 5mm disk of cultivated fungi was taken and mixed with 10 ml sterilized distilled Water [14].

Degradation experiment

One ml of spore suspension was transferred to 250 ml flask containing 100 ml Nette medium with rubber. Control flask has been prepared contain media and rubber only without any fungal species. Flasks were incubated at room temperature with shaking (200 rpm) for 15 days. Three replicates were made.

After 15 days rubbers were taken out carefully, washed thoroughly by distilled water to remove any media components or fungal mycelium if present on their surface and air-dried overnight. The percentage of weight loss was recorded and then sends for scanning electron microscope examination [14].

Laccase and peroxidase detection

Fifteen days fungal culture under shaking and stationary condition at two incubated temperature 28°C and 40°C was filtrated and centrifuged at 10000 rpm for 10 min. The supernatant was used to detect enzyme production [14].

Enzyme assay by Guaiacol assay method

Oxidation of guaiacol has been reported for laccase assay by [20]. The reddish-brown color developed due to oxidation of guaiacol by laccase is used to measure enzyme activity at 450 nm while measure peroxidase activity at 470nm. The reaction mixture can be prepared as follows:

(a) Guaiacol (2 mM) 1 ml.

(b) Sodium Phosphate buffer (10 mM) 3 ml.

(c) Enzyme source 1 ml (fungal supernatant).

A blank was also prepared that contains 1 ml of distilled water instead of enzyme. The mixture was incubated at 30 C for 15 min and the absorbance was read at 450 nm to laccase enzyme and absorbance was read at 470 nm to peroxidase enzyme using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 lmol of guaiacol per min. Manganese peroxidase enzyme activity was calculated by following the laccase enzyme activity determination procedure, but for the reaction mixture 1 mL of H_2O_2 was added and incubated [1]

Scanning Electron Microscopy (SEM) of Rubber

Rubber showed fungal degradation under both 28°C and 40°C after 15 days incubation and that of control rubber (untreated rubber) were examined by scanning electron microscopy (SEM). Specimens for SEM were prepared according to [21], [22].

Results

Isolation of rubber-associated fungi

In this study, four fungal species were isolated from various environmental samples, such as soil and rubber, obtained from Al Shallal in Aswan, and were identified depending on their morphological characters. They belonged to the genus *Aspergillus* (Table 1).

Aspergillus nidulans was isolated from soil while the remaining fungi isolated from rubber tires. Colonies growth at 28˚C was better than 40˚C for all fungal isolates.

Table .1: Rubber associated fungi isolated in Nette's medium at 28˚C **&** 40˚C

strain	Temperature 28˚c	Temperature 40˚c	Source
Aspergillus fumigatus	++	+	rubber
Aspergillus nidulans	++	+	soil
Aspergillus niger	++	+	rubber
Aspergillus oryzae	++	+	rubber

+, <50 colonies/gm; + +, >50 colonies /gm

Genetic characterization using ITS gene sequencing

The ITS1/ITS4 gene was sequenced to identify *Aspergillus nidulans*, *A. fumigatus* and *A. oryzae*. Several sequences were selected from Gene Bank database for the construction of a phylogenetic tree to compare the identified strains (*A. nidulans*, *A. fumigatus* and *A. oryzae*) and with other strains of these species and another genus have similar sequences with these strains.

Nucleotide sequence accession number

The determined sequences of genes for 16S rRNA were deposited at GenBank and are available under following GenBank Accession number: isolate *A. oryzae KhA0707* (OM943825.1), *A. nidulans M93* (PQ624157.1) and *A. fumigatus S73* (PQ624158.1)

16S rDNA sequence analysis

The fungus isolates were identified as *Aspergillus nidulans* (Fig. 2), *A. oryzae* (Fig. 3) and *A. fumigatus* (Fig. 4) using the chromatogram of the ITS gene sequence (Fig. 2), which was confirmed by comparing the ITS gene sequence to known strains in GenBank. *Aspergillus niger* was not sequenced because morphological identification was enough and clear to confirm the identification.

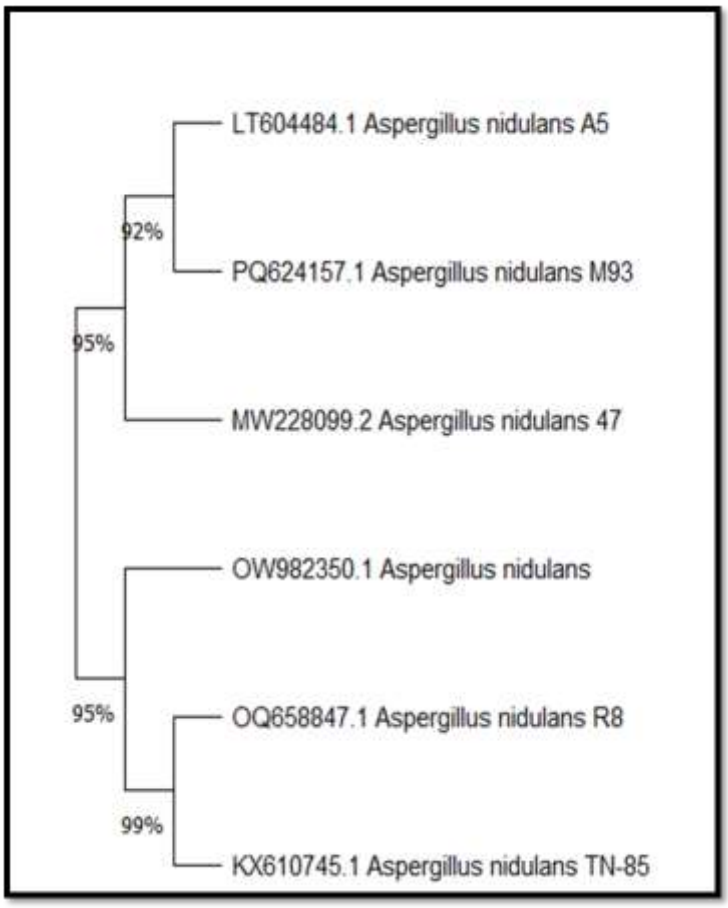

Fig. 2 Phylogenetic tree based on partial ITS sequences, showing the relationship between *A. nidulans M93* and other species. The construction of phylogenetic tree represents Neighbor joining method using MEGA 11 software

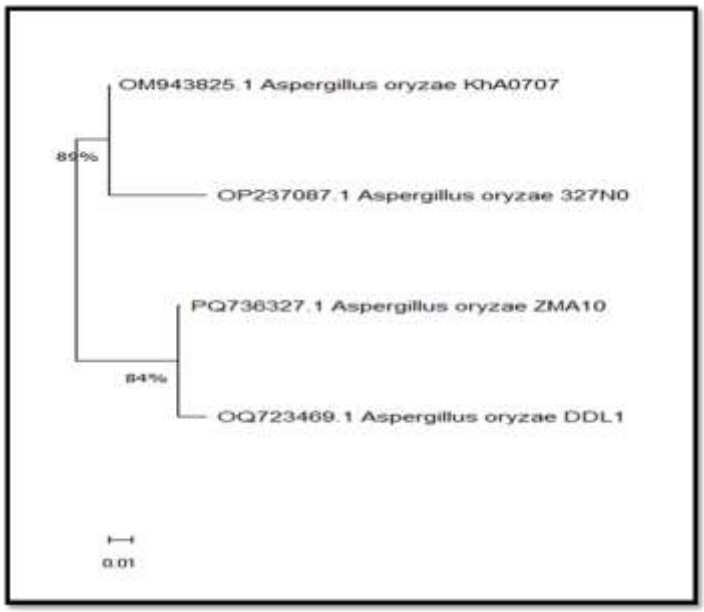

Fig. 3 Phylogenetic tree based on partial ITS sequences, showing the relationship between *A. oryzae KhA0707* and other species. The construction of phylogenetic tree represents Neighbor-joining method using MEGA 11 software

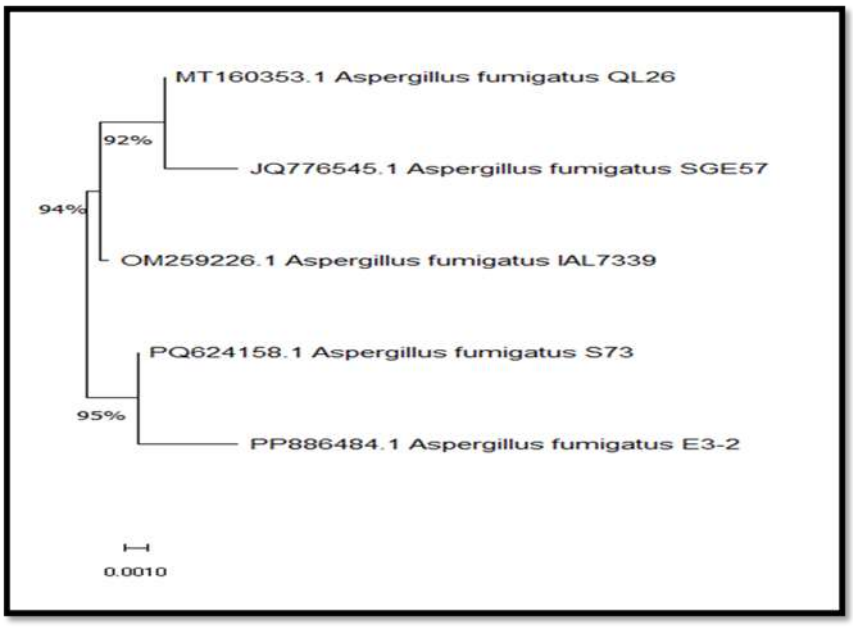

Fig. 4 Phylogenetic tree based on partial ITS sequences, showing the relationship between *A. fumigatus S73* and other species be. The construction of phylogenetic tree represents Neighbor-joining method using MEGA 11 software

Effect of rubber as a sole carbon source in Nette media on the fungal growth weight

Generally, all fungal growth weight on Nette medium showed better growth at 28°C than 40°C (Fig. 5). On the other hand, the media fed with rubber as a sole carbon source recorded better growth than that without rubber at both incubated temperature (Fig. 5). *Aspergillus oryzae* recorded the highest growth weight which was (4.240 and 3.849 gm) at 28°C and (2.841 and 1.905 gm) at 40°C in media with rubber and without rubber respectively. The lowest growth was reported in the culture of *A. nidulans* (2.176 and 1.706 gm) at 28°C and (0.755 and 0.366 gm) at 40 ˚C in culture with rubber and without it respectively (Fig. 5). The growth of *A. fumigatus* was less than that of *A. oryzae* but better than *A. nidulans* and *A. niger* (Fig. 5)

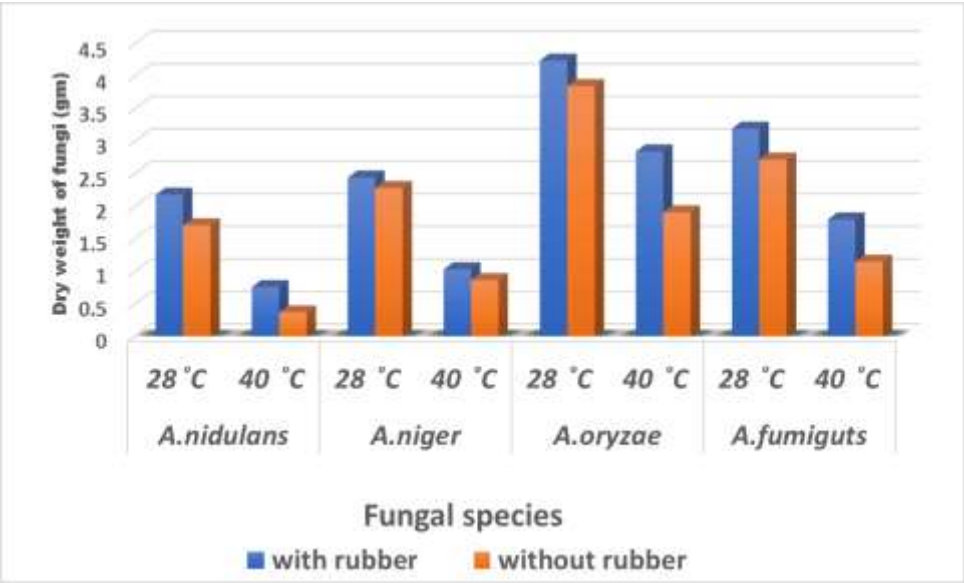

Fig. 5 comparison of the fungal growth in the presence and absence of rubber incubated at 28 ˚C and 40 ˚C

Fungal peroxidase and laccase production in the presence and absence of rubber under shacking conditions at 28 ˚C and 40 ˚C

Aspergillus nidulans, A. niger, A. oryzae, and *A. fumigatus* were cultured in a production medium to study the effect of incubation period and temperature on peroxidase and laccase production using the guaiacol assay method. All isolates produced both enzymes (Figs. 6&7) and this production was enhanced in the presence of rubber at both temperatures. The highest production of laccase was recorded with *A. niger* at 28 ˚C (2.05 U/ml) in rubber fed culture. While fungal incubation at 40 ˚C produced the highest concentration of laccase at this temperature (4.352 U/ml) by *A. oryzae* in rubber treated culture (Fig. 6). Peroxidase production recorded its highest level (11.471 & 9.651 U/ml) by *A. nidulans* at both 28 ˚C and 40 ˚C respectively in culture treated with rubber (Fig. 7).

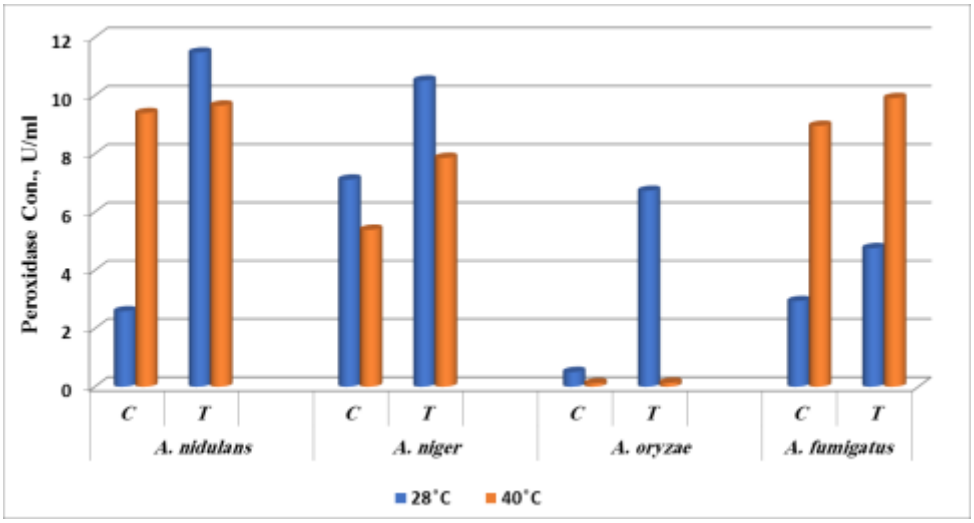

Fig.6 Effect of incubation temperature on peroxidase enzyme production by studied fungi during 15 days at 28˚Cand 40˚C in control culture (C) that do not contain rubber (media +fungi) and culture fed with rubber (T) (media +fungi +rubber)

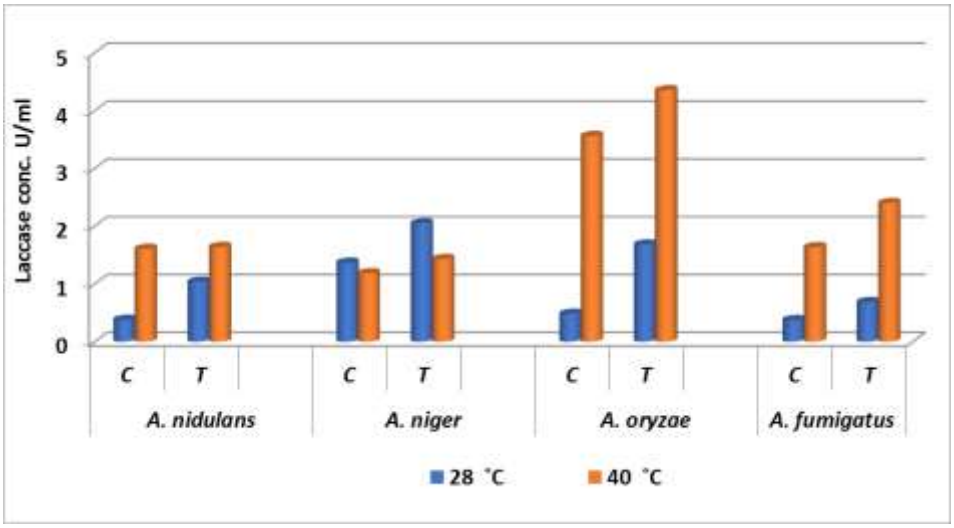

Fig. 7 Effect of incubation temperature on peroxidase enzyme production by studied fungi during 15 days at 28˚Cand 40˚C in control culture (C) that do not contain rubber (media +fungi) and culture fed with rubber (T) (media +fungi +rubber)

Degradation of rubber by the studied fungi

As shown in the results obtained in Fig. 8, the growth experiment was carried out using Nette media. Rubber weight loss and fungal development were detected. At 28°C, natural rubber discs lost 25.76% of their weight when inoculated with *A. fumigatus*, while at 40°C, the weight loss was 3.23%. The results also showed that natural rubber discs inoculated with *A. nidulans* at 28°C showed weight loss 43.67%, while at 40°C it was 31%. *Aspergillus niger*, caused 40% rubber degradation at 28°C, but at 40°C their weight decreased by 15%, while rubber showed weight loss at 28°C by 27% and at 40°C by 13% when inoculated with *A. oryzae*.

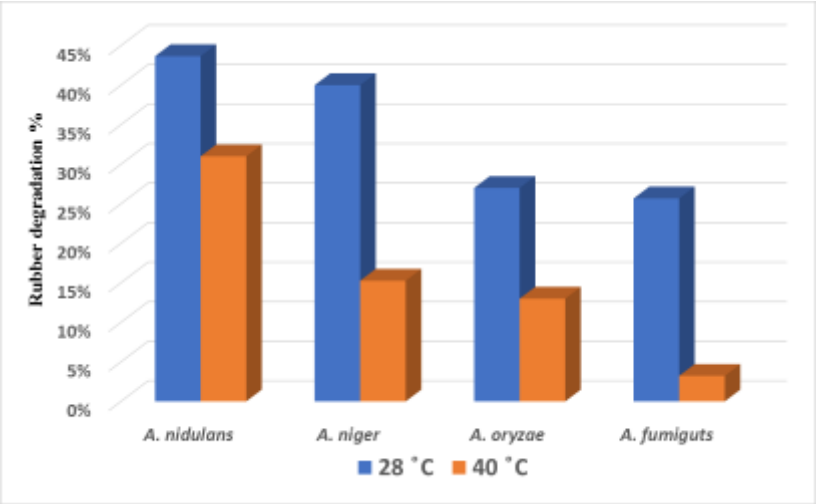

Fig. 8 Degradation percentage of rubber by fungi at 28˚C and 40 ˚C at 15 days

Scanning electron microscope (SEM) of rubber surface

The degradation behavior of each of the selected strains was examined using SEM as a result of hypha formation, rubber disintegration, and biofilm formation. Generally, abundant of all studied fungi on the surface of rubber, clear biofilm formation and disintegration were clear on rubber treated with fungi comparing with control (media with rubber and without any fungus) at both temperatures 25 and 40 ˚C (Figs. 9-16). These phenomena were clearer at 28 C than 40 C which is consistent with the degradation percentage of rubber at both temperatures. The adhesively growing strains *A. nidulans, A. niger,* and *A. oryzae* showed considerable differences from strain *A. fumigatus*. (Figs 9-16) showed the formation of fungal hyphae on rubber by *A. nidulans* cells after 15 days incubation at 28°C. The cells produce special craters on the surface and penetrate the material. A comparable behavior was seen for *A. nidulans* cells followed by *A. niger* after 15 days at 28°C.

Discussion

The disposal of damaged tires represents a critical environmental issue, contributing approximately 12% of global solid waste [23]. Global rubber production has steadily increased, reaching 24.3 million tonnes in 2010, an 11.9% rise from the 21.7 million tonnes produced in 2009. In Egypt alone, an estimated 20 million tires are produced annually, exacerbating the problem of tire waste management. Traditional disposal methods, including storage, burial, and incineration, have significant limitations: storage requires large land areas and fosters pest infestations, burial takes centuries due to the high sulfur content of tires, and incineration generates toxic and carcinogenic compounds that negatively impact air, water, soil, and human health.

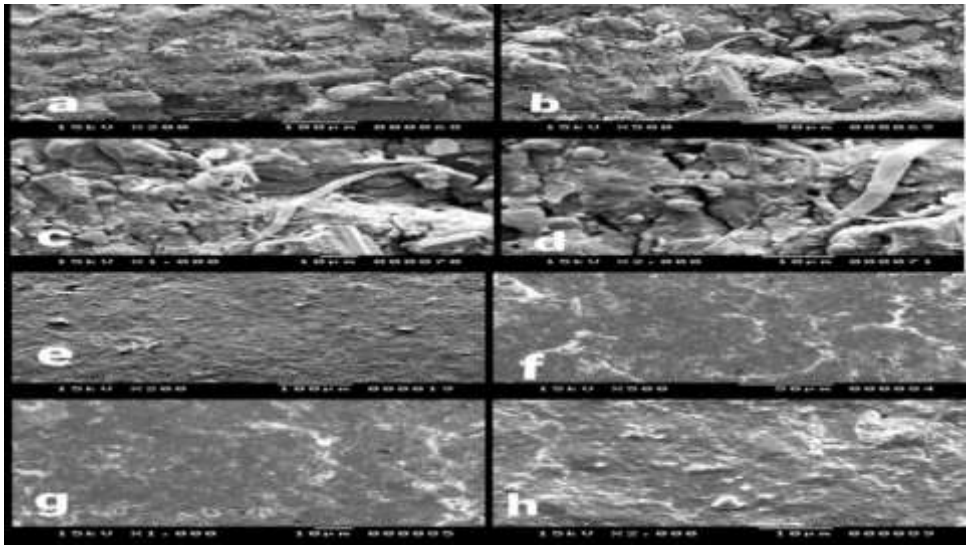

Fig. 9 Scanning electron microscope of rubber treated with *A. nidulans* at 28˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

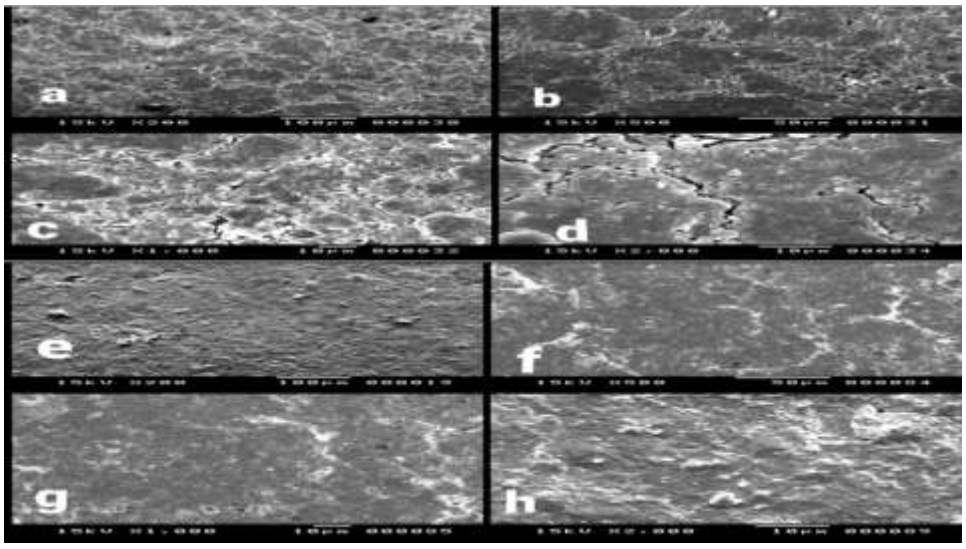

Fig. 10 Scanning electron microscope of rubber treated with *A. nidulans* at 40˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

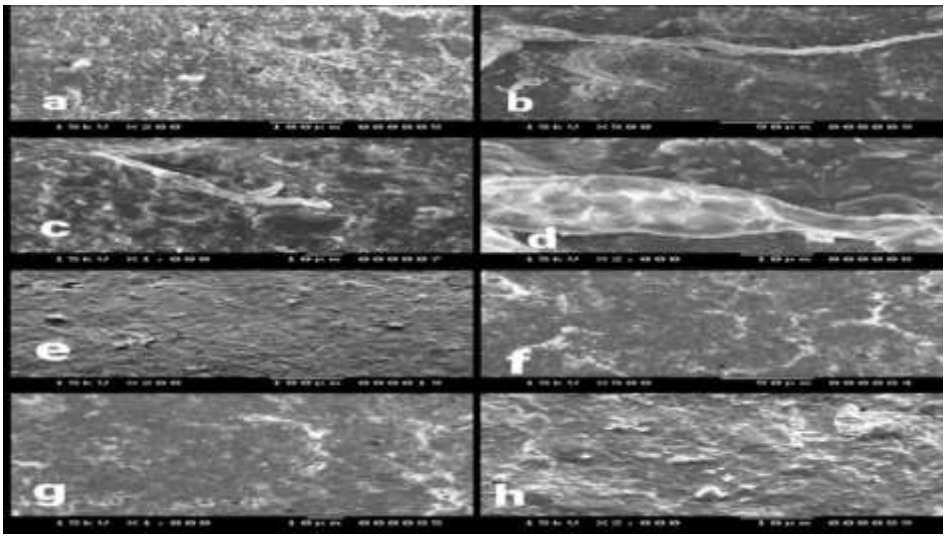

Fig. 11 Scanning electron microscope of rubber treated with *A. niger* at 28˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

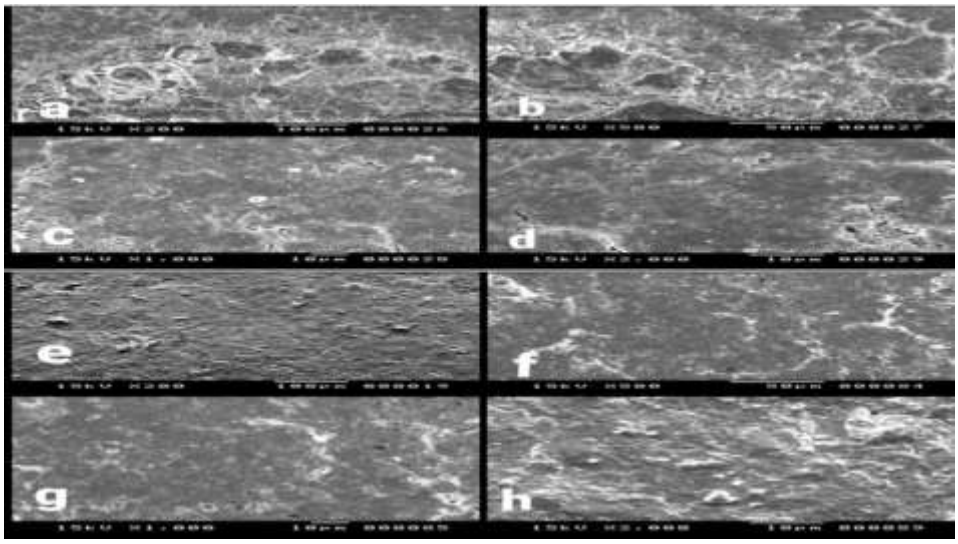

Fig. 12 Scanning electron microscope of rubber treated with *A. niger* at 40˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

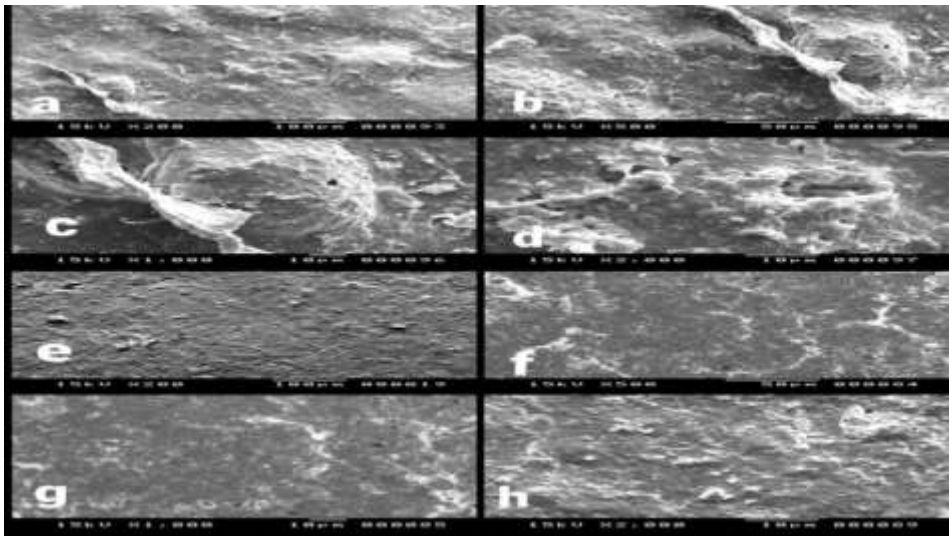

Fig. 13 Scanning electron microscope of rubber treated with *A. oryzae* at 28˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

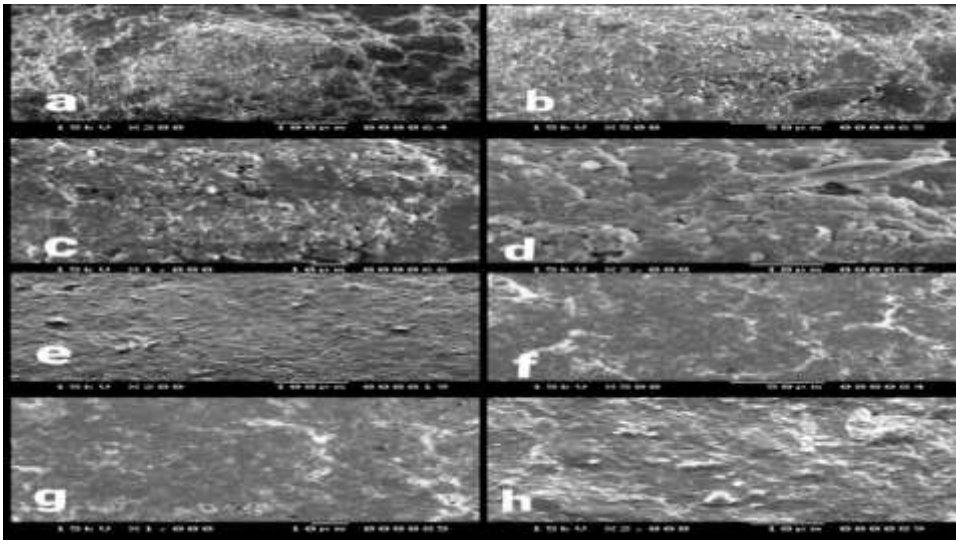

Fig.14 Scanning electron microscope of rubber treated with *A. oryzae* at 40˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

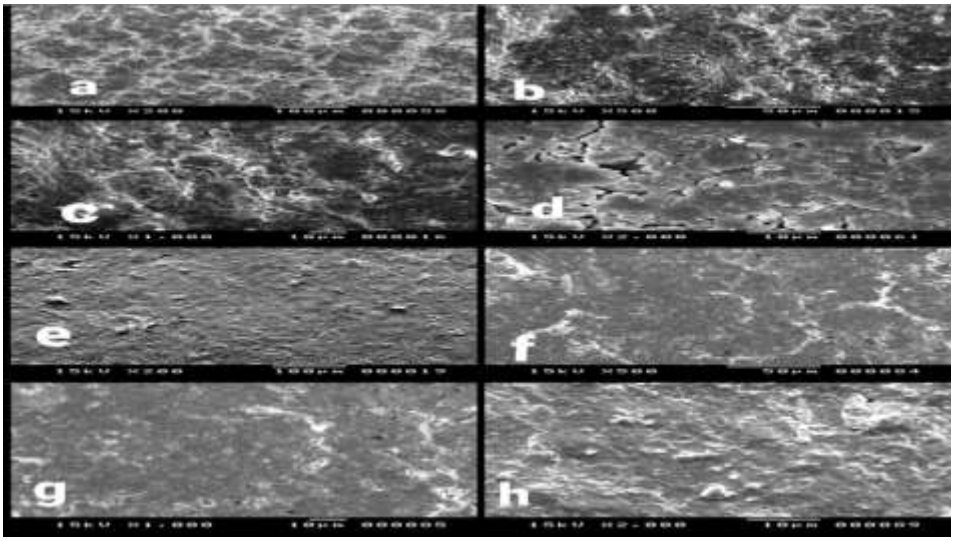

Fig. 15 Scanning electron microscope of rubber treated with *A. fumigatus* at 28˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

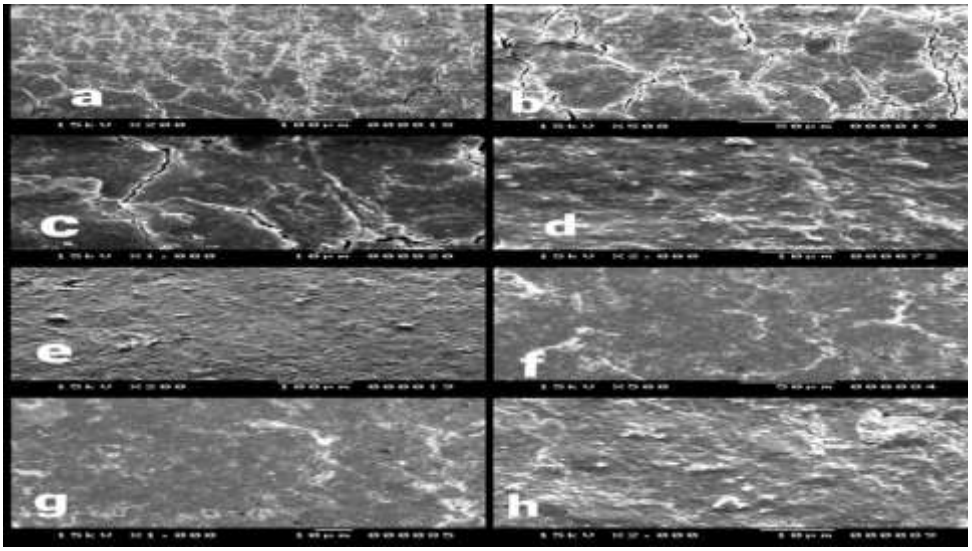

Fig. 16 Scanning electron microscope of rubber treated with *A. fumigatus* at 40˚C (a, b, c, d) comparing with control (e, f, g, h) using different magnifications

Given these challenges, the use of microorganisms for rubber waste management has emerged as a promising, sustainable alternative. Microorganisms are abundant, cost-effective, and capable of degrading rubber efficiently. In the present study, rubber-degrading fungi were isolated using Nette's medium. Rubber pieces embedded in the nutrient medium and incubated at 28°C for 15 days revealed the presence of fungi from the genus *Aspergillus*. This finding is consistent with earlier studies that have demonstrated the ability of fungi to degrade rubber under controlled conditions. There is a diverse fungal species which is capable of degrading rubber. [24] identified *Fusarium solani* as a rapid rubber degrader, surpassing the capabilities of *Paecilomyces lilacinus*, *Phoma eupyrena*, and *Cladosporium cladosporioides*. Similarly, [25] found that *A. niger* and *Phlebia radiata* could degrade natural rubber (NR), with *A. niger* exhibiting the highest degradation capacity. [26] demonstrated that *Alternaria alternata* and the yeast Rhodotorula mucilaginosa could effectively biodegrade NR.

Historical studies also support the role of fungi in rubber degradation. [27] was the first to document fungal decomposition of rubber hydrocarbons, and [28] identified *Aspergillus* and *Penicillium* as effective rubber-degrading genera. Subsequent studies have expanded this knowledge, identifying additional fungal genera, including *Monascus*, *Fusarium, Phoma, Paecilomyces, and Cladosporium*, with rubber-degrading capabilities [29]. Endophytic fungi have also been shown to degrade rubber [30].

In the present study, the concentration of peroxidase in all fungal culture was higher than laccase. Therefore, it seems that peroxidase has a main role in rubber degradation whereas, *E. nidulans* recorded the highest producer for peroxidase in both temperatures and the degradation was the highest also which realized by the degradation percentage and the examination using SEM.

Phylogenetic trees are a type of visualization that depicts abstract concepts rather than their appearances (iconic diagrams) or quantitative correlations [31]. To understand phylogenetic trees, it is required to acquire standards, get over previous, frequently elementary knowledge of taxa, and evaluate evolutionary relationships based only on branching patterns displayed in the diagrams [32][33].

Conclusion

Natural rubber (NR) is an important polymer found in more than 40,000 products, including medical equipment, surgical gloves, tires, pacifiers, clothing, and toys. Synthetic polyisoprene, with purity ranging from 98 to 99%, has physical qualities similar to NR but lacks stress stability. Scientists have investigated microbial degradation methods for breaking down NR, which are more environmentally benign than chemical and physical breakdown. Fungi have been investigated for their potential to degrade NR since 1928, although the majority of study has concentrated on the foundations of rubber biodegradation. The understanding of enzyme action on rubber substrates, fungal, molecular and environmental variables are inadequate. Continuous research is required to create scalable NR biodegradation techniques and systems.

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