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Eco-Friendly Chitosan Polymer Synthesis Using *Rhizopus Arrhizus* RL Strain and Evaluation of its Antibacterial Activities

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Abstract

Chitosan is found in the cell structure of Zygomycetes fungi. It is a deacetylated chitin derivative, nature's second most abundant polysaccharide after cellulose. The current proposal used antimicrobial fungal chitosan polymer substances against foodborne bacterial pathogens. Ten fungal isolates were isolated from soil in the present investigation, and chitin was extracted from the fungal cell wall to produce chitosan. These isolates belonged to the *Rhizopus, Aspergillus, Trichoderma, and Penicillium genera. Rhizopus sp*. code RL was the most effective isolate regarding mycelium dry weight (1.05g/L) and chitin and chitosan yields (0.86 g/g mycelium and 0.12 g/g chitin). The chitosan productivity and yield coefficients relative to mycelium dry weight were 0.03 g/L/d and 0.11 g/g/L, respectively. 18S rRNA sequencing-based phylogenetic analysis revealed that this isolate had a 100% similarity to *Rhizopus arrhizus* for molecular identification. The gene sequences identified during the current study have been submitted to the GenBank database under the accession number OR398886. The fungal chitosan polymer chemical structure was identified using ^{1}H nuclear magnetic resonance. In addition, the tested polymer was used as a natural antibacterial agent against foodborne pathogenic bacterial strains. The results showed that *Staphylococcus aureus* As4 and *Salmonella typhimurium* As3 were more susceptible to the fungal polymer, with inhibition zones measuring 24 and 15 mm and minimum inhibitory concentrations of 2.5 and 1.25 mg/mL, respectively. The tested polymer did not exhibit any cytotoxicity in the cell line withIC_{50} of 367.49±7.16 µg/ml. The present investigation revealed that *Rhizopus arrhizus* RL chitosan polymer can be used as a potentgreen-friendly polymerto preserve food.

Keywords: Chitin extraction; Chitosan production; Fungal isolation; Molecular characterization; Polymer identification

1. Introduction

One of the many types of microorganisms is a fungus, which lives in a variety of environmental environments, including soil, industrial corridors (leaves, roots, and fruits), water, and food sources [1,2,3] Environmental parameters such as temperature, pH, humidity, degree of aeration, quantity, and type of nutrients all impact the growth and distribution of fungus [4]. Both chitin and its branch chitosan are naturally formed polysaccharides formed of the mono-sucrose N-acetyl-D-glucosamine and D-glucosamine and are linked together by 1,4-glycoside linkages. The patch can be chitin or chitosan, depending on how frequently the last monosaccharides exist. Chitin can be turned into chitosan by partially deacetylating the monomer N acetyl D glucosamine, which naturally occurs as a critical component in the cadaverous or exoskeletal structures of lower species [5]. Chitin contains a significant amount of N acetyl D glucosamine. Several studies on chitosan are made from various fungi mycelium. The second-most abundant component of biomass on earth, chitin is also an essential component of the fungal cell structure [6]. Chitin is a very difficult substance that has yet to be used in a significant artificial capacity. Rouget noted

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the discovery of chitosan in 1859 after chitin was boiled in potassium hydroxide.

The substance was now acid-soluble after this treatment. Additionally, [7] examined the ability of chitosan to inhibit the growth of food-corruption bacteria (*Lactobacillus plantarum* and *L. fructivorans*, *Serratia liquefaciens*, and *Zygosaccharomyces bailii*) in mayonnaise.

Synthetic chemicals such as nitrates, nitrites, sulfites, sodium benzoate, propyl gallate, and potassium sorbate are traditional preservatives. Due to research on headaches, aversions, and cancer, artificial preservatives are being revaluated. As the demand for biopreservation in food systems has increased, new natural antimicrobial composites of colourful origins are being developed, including factory-deduced products (polyphenolics, essential canvases, factory antimicrobial peptides, beast-deduced products (lysozymes, lactoperoxidase, lactoferrin, ovotransferrin, antimicrobial peptide, and microbial metabolites (nisin, natamycin, pullulan, ε-polylysine, organic acid, and others). These organic preservatives prevent the replication of DNA and RNA, recapitulation, protein conflation, and metabolism in microorganisms [8,9]. The safety of natural preservatives has received confirmation. Still, although being efficient food preservatives, they can have a significant impact on color, fragrance, and toxicity.

In order to assess the antibacterial activity and minimal inhibitory inhibition of chitosan polymer, the aim of the present research was to protect different fungal species from its conflation.

2. Materials and Methods

2.1. Collection of samples

Mud samples were taken from the Faculty of Agriculture Farm, Qalyubia Governorate, Egypt, in May 2020. The samples were divided and classified in accordance with the location of their collection. Mud samples were taken at a depth of 10 cm near roots where most of the microbial activity was concentrated from different areas. These samples were procured in sterile plastic bags and put in an ice box in a research facility [4].

2.2. Pathogenic bacterial strains

Six bacterial pathogenesis strains were purchased from Microbiological Resource Centers (MIRCEN), Cairo, Egypt. These strains were *Escherichia coli* O157H7,*Staphylococcus aureus* As4,*Salmonella typhimurium* As3, *Shigella shag* As4, *Bacillus cereus* DSMZ345, and *Listeria monocytogenes* DSMZ 423.

2.3. Isolation and purification Fungi

The soil dilution technique described by [10] performed fungal insulation from the soil. For serial dilutions (10^{-1} to 10^{-5}), ten grams of the soil sample were suspended in 90 ml of sterile distilled water. On malt agar, one milliliter of each dilution was applied (its combined g/l malt, 30; incentive excerpt, 5, and agar, 20), according to [11]. The dishes were grown for 3 to 5 days at 28 ± 2°C in the darkness. Additionally, these colonies were moved to different malt agar dishes to get pure colonies and then preserved at 5°C on a malt agar slant for further research after the developing fungi were checked under a light microscope (400 x) to confirm that they were pure as a single spore.

2.4. Maintenance of cultures

Following incubation at 28 ± 2°C for 3-5 days, the cultured stock slants remained at 5°C on a preservation malt agar. Sub-culturing was usually carried out every month using the same medium.

2.5. Morphological and culture identification of fungal isolates

Fungal morphology was studied microscopically, and cultural properties of colony characteristics (colour, shape, size, and hyphae) were observed by necked eyes, according to [12].

2.6. Preparation of spore suspension and standard fungal inoculum

The spore suspensions were made by adding 5 ml of autoclaved tap water to each malt agar slant on which the tested fungal isolates were grown for 3–5 days at 28°C and then gently scraping the surface of the culture with a sterile inoculation loop. The necessary amount of spore suspension for inoculation was created by combining the spore suspensions from each slant. To maintain the conidia distributed, a drop of tween-80 was added to the concentrated spore suspension before being transferred to a conical flask filled with distilled water and thoroughly shaken for 10 min. A sterilized filter paper (Whatman No. 1 double ring) filters the suspension. The spores were counted using a Neubauerhemocytometer slide 8 x10^6 [13].

2.7. Fungal biomass

The cultures were inoculated in 250 ml conical flasks to produce fungal mycelium, making 100 ml from malt broth medium [11]. Standard inoculum of 1 ml fungal spore suspension and kept there for 3-5 days at 28±2°C. The fungal biomass (mycelium) was filtrated using filter paper (Whatman No. 1 double ring). The mycelium on the filter had been washed with distilled water around three times

before being dried in a Heraeus oven at 80°C until it reached a consistent weight.

2.8. Extraction of chitin from isolated fungal isolates

The dried mycelium (fungal biomass) was extracted using the procedure described by**[5].**Weighing the dried mycelium, 1 g was extracted and treated using 30 ml of sodium hydroxide (40%) solution in a conical flask. Proteins, lipids, and alkali-soluble polysaccharides included in the mixture were removed by incubating at 121°C for 15-20 min. The mixture was then centrifuged at 12000 *xg* for 15 min to collect the alkaline-insoluble chitin. The precipitate was doubly rinsed with ethanol (95%), dried in a hot oven at 60°C for 24 h, and the supernatant was removed. The dry mass of the chitin was determined.

2.9. Chitosan production

The chitin dry weight produced was converted to chitosan. One gram of dry chitin was added to a conical flask and treated with 40 ml of 2% acetic acid for 8 h at 95°C. The pH was brought up to 10 using 2M sodium hydroxide after 8 h. The alkali-insoluble chitosan precipitation was centrifuged at 12000 x g for 15 min, then washed using sterilized water, dried, and then measured [5]. The polymer kinetics of productivity [14] and yield coefficients relative to mycelium dry weight [15] were calculated according to the following equations (1 and 2):

Chitosan productivity (g/l/d)=Chitosan dry weight (g/g mycelium dry weight/l)/Fermentation time (day) (1)

Chitosan yield coefficients relative to mycelium dry weight (g/g/l)=Chitosan dry weight (g) / Mycelium dry weigh (g/l) (2)

2.10. Molecular identification of the utmost colonist fungal insulate for fungal chitosan production

The fungus insulation was included with sterile Petri dishes with a 9 cm diameter and 20 mL of potato sucrose agar and then incubated for 5 days at 28±2°C [16]. The cell culture was given to the Molecular Biology Research Unit at the Assiut University's Faculty of Science in Egypt's Assiut. Patho-gene-spin DNA/ RNA birth tackle provided by the Korean company Intron Biotechnology Company was employed for DNA synthesis. Prior to being sent to SolGent Company, Daejeon, South Korea, for polymerase chain reaction (PCR) and rRNA gene sequencing, the uprooted DNA was maintained in a 1.5 ml autoclaved Eppendorf tube. The ITS1 (forward) and ITS4 (reverse) manuals included with the response mixtures were used to do the PCR. Manuals are made up of the following: ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') and ITS1 (5'- TCC GTA GGT GAA CCT GCG G- 3'). With the addition of ddNTPs in the response admixture, the purified PCR product was sequenced using the same protocols [17]. The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website. MegAlign (DNA Star) software interpretation 5.05 was used to perform phylogenetic analysis on sequences.

2.11. Identification of fungal chitosan polymer using ^{1}H nuclear magnetic resonance (^{1}H NMR) assay

This polymer was linked grounded on protons using a method described by [18]. ^{1}H NMR spectra of sample was recorded using Jeol 500 MH spectrometer JAPAN in D_2O result. Chitosan samples were diluted to a concentration of 5 mg/cm^3 in a waterless deuterated acid called TFF/D_2O, which has a pH of about 4. In a plastic tube, chitosan weighing 0.05 g was weighed. The samples were then each given 6 cm^3 of D_2O and 1 cm^3 of deuterium chloride to obtain results. To an end quantity of 10 cm^3 and pH of ca, D_2O and TFF was added 4. To replace labile protons with deuterium titers, the materials were twice indurate-dried using D_2O (99.9). Three samples of each type of chitosan were measured. Spectra between 0 and 6 ppm were collected at 27°C and 60°C using the NMR spectrometer Bruker Avance II (700 MHz). The degree of deacetylation is determined using integrals under typical signals.

2.12. Evaluation of the antibacterial activity of fungal chitosan polymer and antibiotics

This experiment was performed in order to study the fungal chitosan polymers and antibiotics described by the Clinical and Laboratory Standards Institute (CLSI) inhibitory effect against some pathogenic bacterial strains. It was detected using the well diffusion agar technique [19]. The experiment was conducted to grow pathogenic microbes in a tryptone soy broth medium at 37°C for 24 h. One thousand microliters of pathogenic bacteria suspension into sterile plates, then 10 mL of tryptone soya agar medium, allow the surface of the medium to dry for 5 min. A hole was made in the dried agar by sterile cork borer, and 100 µg of the polymer antibacterial agent was applied. Antibiotic of was used as a positive control. The dishes stay incubated for 24 h at 37°C. Measurements of the inhibition zone diameter (IZD) around the growing colonies were used to test the antibacterial activity. The antibacterial index was calculated.

Antibacterial activity percentage= $\left(\frac{\text{Polymer IZD (mm)}}{\text{Antibiotic IZD (mm)}}\right) X100$

2.13. Determination of minimum inhibition concentration (MIC) by well diffusion agar plate

The minimum inhibition concentration (MIC) test was carried out to evaluate the fungal chitosan polymer's antibacterial activity by two-fold dilutions through the well diffusion agar plate [19]. The MIC of fungal chitosan polymer was identified to determine the lowest concentration inhibiting the test organisms' visible growth. One milliliter of different concentrations of the fungal chitosan polymer (10, 5, 2.5, 1.25, and 0.625 mg/mL) was added to the intact plates. All the plates were incubated at 37°C for 24 h to determine the inhibitory growth of fungal chitosan polymer on a particular pathogen.

2.14. Determination of sample cytotoxicity on Vero cells (MTT protocol)

The Vero cell line (a normal kidney CCL-81) from the Chlorocebus aethiops organism was used to investigate the cytotoxic effect of the fungal chitosan polymer using the MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which was described by [20]. At the Science Way for Scientific Research and Consultations Company in Cairo, Egypt, this experiment was carried out. A full monolayer sheet was formed in the 96-well tissue culture plate after 24 hours of incubation at 37°C with 100 L/well of standard cell viability ($1x10^5$ cells/ml). The growth medium was removed from 96-well microtiter plates after the confluent sheet of cells had formed, and the cell monolayer was then twice washed with wash media. In RPMI medium containing 2% serum, two-fold dilutions of the fungal chitosan polymer were created (maintenance medium). Three wells were utilized as controls and received nothing but maintenance medium while the other wells each received 0.1 ml of each dilution for testing. The plate was tested after being incubated at 37°C. (5 mg/ml in PBS) MTT solution was made (BIO BASIC CANADA INC.). Each well received 20 microliters of MTT the solution, which was then mixed with the media by shaking the wells at 150 rpm for five minutes. The cells were then left to metabolize the MTT for 4 hours at 37°C and 5% CO_2. To combine the formazan and the solvent, resuspend the formazan (an MTT metabolic product) in 200 L of dimethyl sulfoxide (DMSO) and shake at 150 rpm for five minutes. The optical density was calculated as the cell amount from the absorbance at 560 nm for each well in a microtiter plate reader.

After being exposed to a fungal chitosan polymer, morphological cells from cytotoxicity research were inspected for any outward indications of harm. The alterations include rounding, shrinkage, partial or full monolayer loss, and cell granulation.

2.15. Statistical analysis

Data were statistically analyzed using IBM® SPSS® Statistics program version 19 based on Duncan's Multiple Range Test [21] at 5%.

Result and discussion

3.1. Isolation and phenotypic identification of fungal isolates

In this experiment, ten fungal isolates were isolated from soil samples on malt agar medium at 28±2°C for 3-5 days of incubation periods. The isolate codes were RL, A1, A2, A3, An, P1, P2, P3, T1, and T2. These isolates were identified based on culture, and morphological properties have been shown in Fig. (1a and b). Out of 10 isolates, 4 fungal isolate codes of A1, A2, A3, and An belonging to the genus *Aspergillus* were obtained. The cultured characteristics of these isolates (Fig. 1a) formed black, flat-growing colonies on malt agar medium. The growth begins getting white, but after a few days, it turns black and releases a conidial spore. Under a light microscope, the isolate had the morphology of a filamentous fungus, producing filamented hyphae that gave them the appearance of miniature plants (Fig. 1b). One fungal isolate, coded RL, had a colony with a profoundly cottony structure with a top that was white to grey and a reverse that was pure white.

When examined under a light microscope, the isolate has a filamentous appearance. Broad hyphae that are Nonseptate or only sparsely septate, sporangiophores, rhizoids (hyphae that resemble roots), sporangia, and sporangiospores can all be seen. Sporangiophores are often unbranched and brown in colour. They may operate alone or in groups. In addition, 3 fungal isolate codes P1, P2, and P3 were initially white and became green, grey-green, and soft with time. Each branch resulted in a conidiophore, accompanied by conidia, a shade of green spherical, constricted units. Conidia carriers produce conidia, responsible for forming conidia spores (Fig. 1a and b). Two fungal isolates (T1 and T2) were characterized, where the fungal colony appears to have a light or compact appearance, and the conidia spores give a white mycelium. The green colour seems to be on the aerial parts of the mycelium. The mycelium appears under a light microscope as a complex of yellowish hyphae, divided, branched, with smooth walls, conidia, conical or pyramidal, many-branched, bearing

phalloids in the form of flagella, which in turn carry conidia.

Moreover, Udoh et al. [22] the post-harvest rotting of several edible fruits and vegetables was caused by various fungal species isolated from those same fruits and vegetables. In addition, Yang et al. [23] found a fungal mycelium source of chitosan. *Rhizopus Oryza* was extracted to extract chitosan from according to Chatterjee et al. [24]. According to some recent studies, Chitosan can be extracted from fungus [25]. Several fungi, including *Rhizopus oryzae, Mucor rouxii, Gongronella butleri, Aspergillus niger, Trichoderma reesei, Penicillium verruculosum, and Penicillium chrysogenum,* have been observed to be composed of chitin and chitosan in the structure of their cell walls [26,27,28].

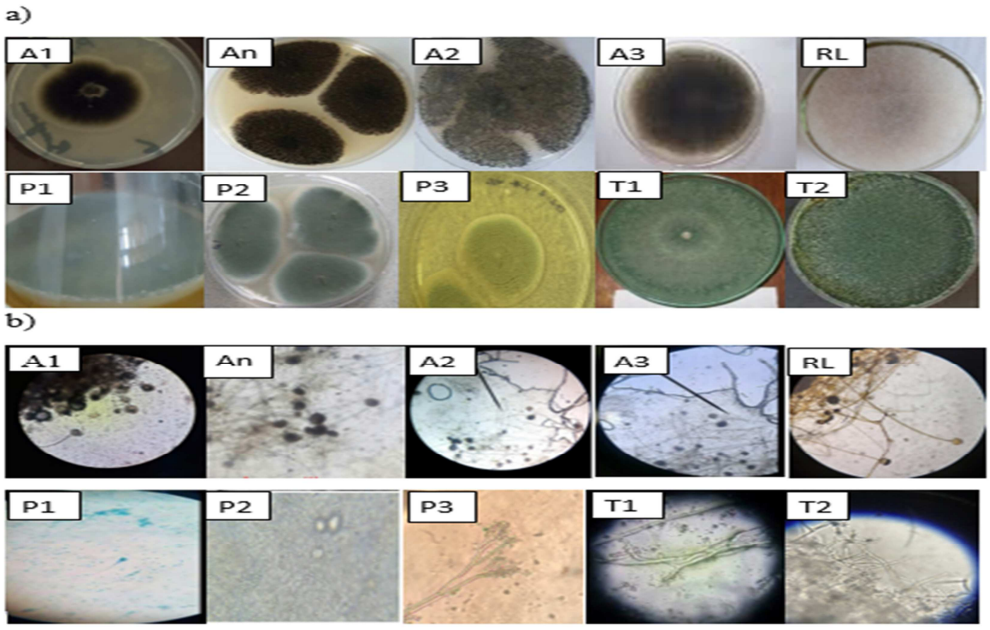

Figure 1: Phenotypic identification, (a) cultural characterization on a malt agar plate, (b) morphological characterization under a light microscope (400 X).
A1, An, A2, and A3 isolate codes belonging to the genus *Aspergillus*, RL isolate code belonging to the genus *Rhizopus*; P1, P2 and P3 isolate codes belonging to the genus *Penicillium*; T1 and T2 isolate codes belonging to the genus *Trichoderma.*

3.2. Extraction of chitin and chitosan production from isolated fungal isolates

Results in Fig. (2 a-c) extremely showed that the fungal isolates dry weight and the amount of chitin and chitosan. Results in Fig. (2a) exhibited that the maximum significant ($p \leq 0.05$) mycelial yield was obtained with *Rhizopus* sp. code RL (1.05 g/l) followed by *Aspergillus* sp. codes A1 and A2, which produced 0.81 and 0.83 g/L of mycelium dry weight. Whereas the mycelial dry weights (0.79 g/l) of *Aspergillus* sp. code An, *Penicillium* sp. codes P1 and P2 were not difference significant. While *Trichoderma* sp. codes T1 and T2*, Aspergillus* sp. code A3, and *Penicillium* sp. code P3 recorded the least amounts of mycelium dry weight, which increased between 0.10 and 0.12 g/l.

After KOH treatment, chitin was extracted, cleared with concentrated ethanol, dried, and its dry weight was calculated. Data in Fig. (2b) showed the chitin dry weight from the fungal isolates ranged from 0.03 to 0.86 g/g mycelium dry weight. *Rhizopus* sp. code RL,and *Aspergillus* sp. codes A1 and A2 recorded the maximum significant ($p \leq 0.05$) chitin yield of 0.86,0.77, and 0.79 g/g mycelium dry weight, respectively. This rise can be attributed to the time-dependent increase in chitin content of the cell wall in *Rhizopus* sp. code RL. In addition, the fungal isolates *Aspergillus* sp. code An and *Penicillium* sp. codes P1 and P2 were achieved0.67, 0.46 and 0.68 g chitin/g mycelium dry weight, respectively. While the lowest chitin dry weight was recorded with other fungal isolates, increased between 0.33 and 0.88 g chitin/g mycelium dry weight.

The dry mass of chitosan was estimated and represented in Fig. (2c). *Rhizopus* sp. code RL produced the highest significant ($p \leq 0.05$) amount of chitosan, 0.120 g / g of mycelium on a dry mass basis, followed by *Aspergillus* sp. code An and A1, having 0.065 and 0.058 g /g of mycelium (not difference significant). The fungal isolates *Aspergillus* sp. codeA2, *Penicillium* sp. codes P1 and

P2 and *Trichoderma* sp. codes T1 and T2 were recorded the chitosan dry mass increased between 0.028 and 0.042 g chitin/g mycelium dry weight, respectively. The lowest amount of chitosan (0.017 and 0.016 g /g of mycelium) was exhibited with *Aspergillus* sp. code A3 and *Penicillium* sp. code P3, respectively.

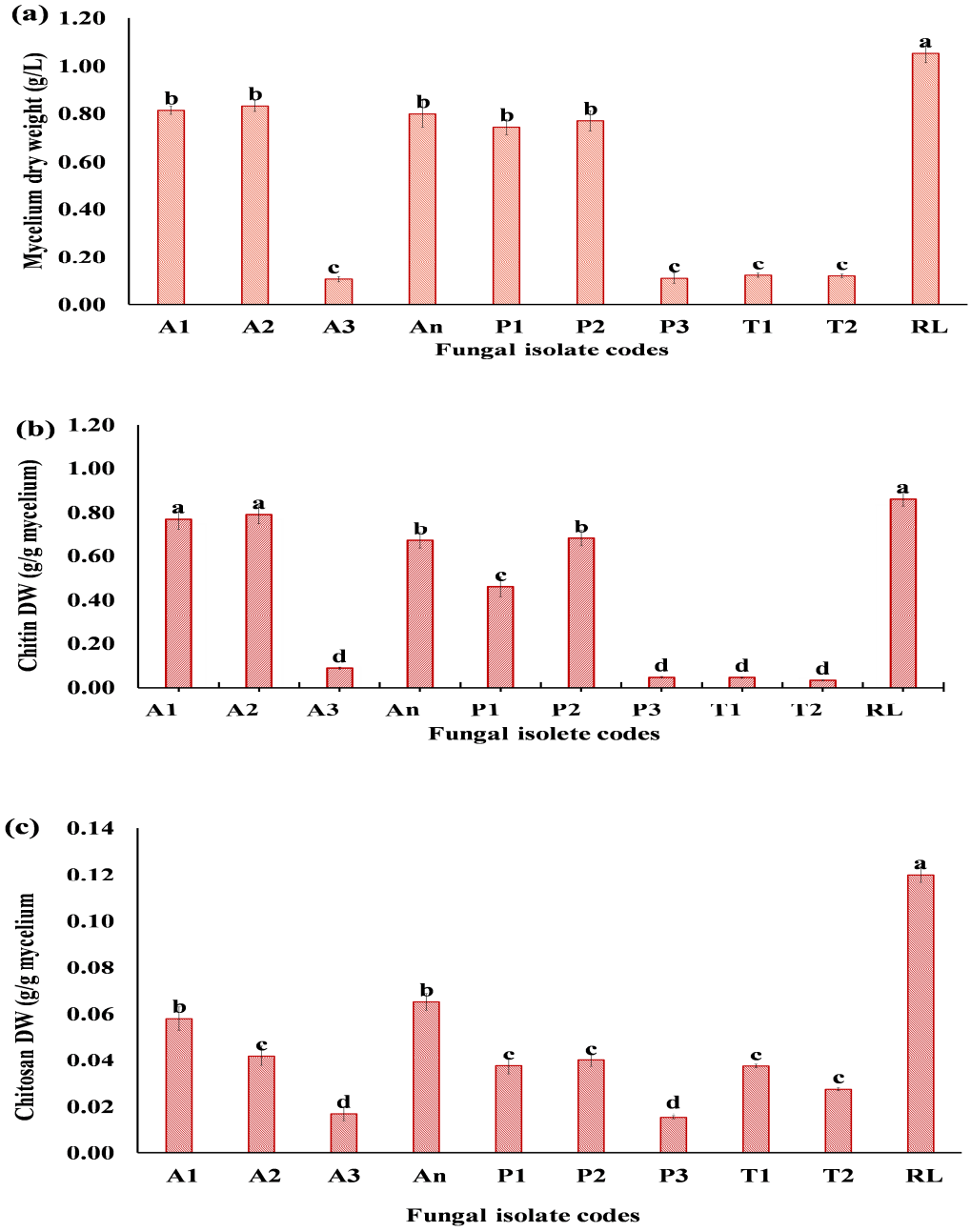

Figure 2: Cell dry weight (a), chitin dry weight (b), and chitosan dry weight (c) synthesized from fungal isolates.
A1, An, A2, and A3 isolate codes belonging to the genus Aspergillus, RL isolate code belonging to the genus Rhizopus; P1, P2 and P3 isolate codes belonging to the genus Penicillium; T1 and T2 isolate codes belonging to the genus Trichoderma.
[a, b] Means values with small letters above the column followed by different letters are significantly different at p< 0.05 level. Bar indicated to± standard division (SD)

From the above results, 3 fungal isolates among 10 isolates were chosen for chitosan production. These isolates were *Rhizopus* sp. code RL, *Aspergillus* sp. code An, and *Aspergillus* sp. code A1. The kinetics parameters of the most efficient fungal chitosan polymer produced by the 3 selected fungal isolates were calculated and tabulated in Table 1. The chitosan yield coefficient relative to mycelium dry weight was 0.11, 0.08, and 0.07 g/g/L for *Rhizopus* sp. code RL, *Aspergillus* sp. code An, and *Aspergillus* sp. code A1. Moreover, the highest chitosan productivity (0.03 g/g/L) was achieved by *Rhizopus* sp. code RL. Whereas the productivity of both *Aspergillus* sp. codes A1 and An were 0.015 and 0.016 g/L/d.

As mentioned previously in this study, the first step in fungal chitosan extraction was the deproteination step using 1M NaOH for 15 minutes at 121°C. This step breaks down the cell wall, releasing the internal components and extracting free chitosan as well [29,30]. Moreover, [31] reported that 2.36 g/L of fungal biomass and 9.3 g/L of lactic acid were found in the broth after 72 h, though the final product of chitosan extracting applying *Rhizopus oryza* AS 3.81 was 0.11 g/g biomass. In addition, [32] stated that the curve for chitosan production has the same profile as the microorganism's growth curve.Since chitosan was an element of filamentous fungal cell walls, the quantum of chitosan produced increases as the growth increases upon agitation. The precursor, also called chitosan, chitin, is deacetylated in the fungal cell membrane, and the active growth results in a high ratio of fungal chitosan [33]. According to [34], exopolysaccharide polymers generated by *Stenotrophomonas daejeonensis* B14 and *Pseudomonas geniculate* Y8 were optimized using statistical methods. They excelled standard processes by roughly 1.07 and 1.09 times on a low-cost medium that contained blackstrap molasses and CSL as economic by products. So, the fungal isolate *Rhizopus* sp. code RL was the most effective isolate for producing chitin and chitosan. It was selected for the next study.

Table 1: Kinetics parameters of the most efficient fungal isolates for the production of chitosan

Kinetics parameters	Fungal isolates		
	Rhizopus sp. (code RL)	*Aspergillus* sp. (code An)	*Aspergillus* sp. (code A1)
MDW	1.05	0.8	0.81
Chitin dry weight (g/g MDW)	0.86	0.7	0.77
Chitosan dry weight (g/g chitin dry weight)	0.12	0.07	0.06
$Y_{C/M}$ (g/g/L)	0.11	0.08	0.07
Productivity (g/L/d)	0.03	0.016	0.015

MDW; mycelium dry weight, $Y_{C/M}$; chitosan yield coefficient relative to mycelium dry weight

3.3. Molecular identification of the most pioneer fungal isolate for chitosan production

Molecular identification and classification based on rDNA sequencing analysis is a crucial technique for effectively categorizing microorganisms preceding morphological, physiological, and biochemical characterization Due to its complexity and length. Nucleotide sequence analysis for *Rhizopus* sp. code RL was found to be 623 bp order corresponding to the C-terminal region of the rDNA gene (Fig. 3). The rDNA gene sequence for *Rhizopus* sp. code RL isolate was aligned with 100 identified strains in the NCBI (www.ncbi.nlm.nih.gov) in Gene Bank using the BLAST and the Ribosomal Database Project (RDP) databases under the specified accession numbers. Table (2) shows that the fifteenth strains in the gene bank were significantly aligned with the tested isolate.

A phylogenetic tree was created using a Neighbor-joining tree distance-based algorithm. The isolated fungal strain *Rhizopus* sp. code RL sequence was found to be 100% similar to *Rhizopus arrhizus* strain DTO400-C5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Accession: MT316366.1). The 623 nucleotide 18s rRNA gene analysis was submitted to GenBank under the accession number OR398886 (Fig. 4). As a result, this strain was given the names *Rhizopus arrhizus* strain partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Accession no. OR398886).

Furthermore, according to the published review, the published study, the most successful strains produced were *Mucor mucedo, Phycomyces blakesleeanus, Aspergillus niger, Mucor rouxii,* and *Rhizopus arrhizus* [35,36,37].

***Rhizopus* sp. code RL (Length 623 bp)**

TAGGTGAACCTGCGGAAGGATCATTAATTATGTTAAAGCGCCTTACCTTAGGGTTTCCTCTGGG
GTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAGAGACTCAGACTGGTCATGGGTAGACC
TATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCACCCTTCATAATAAACCTAGAAATT
CAGTATTTAAAGTTTAATAAAAAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAA
GAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAAC
GCAGCTTGCACTCTATGGTTTTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACA
TAACATTTGTTTATGTGGTGATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGT
GTGATTTTCTGTCTGGCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCTTTTTTTTTGGTT
CGCCCAGGAAGTAAAGTACAAGAGTATAATCCAGTAACTTTCAAACTATGATCTGAAGTCAGGT
GGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Figure 3: Nucleotide sequence of rRNA of the fungal isolate of *Rhizopus* sp. (code RL)

Table 2: The accession number of *Rhizopus arrhizus* strains recorded into the gene bank significantly aligned with the selected *Rhizopus*sp. (RL isolate)

Description	Max Score	Total Score	Query Cover	Per. ident	Acc. Len	Accession
Rhizopus arrhizus strain DTO 400-C5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	900	MT316366.1
Rhizopus arrhizus isolate RS3-S2-25 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	655	MN547407.1
Rhizopus arrhizus strain 2ENV 18 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	627	MH681101.1
Rhizopus arrhizus strain NB31 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	785	MF685318.1
Rhizopus oryzae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: IFM 62202	1151	1151	100%	100	651	LC317754.1
Rhizopus sp. BAB-4277 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1151	1230	100%	100	664	KM401403.1
Rhizopus arrhizus strain ZZP3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	651	OM850428.1
Rhizopus arrhizus strain YAP3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	655	OM850424.1
Rhizopus arrhizus isolate 103 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	630	MW785831.1
Rhizopus arrhizus isolate 86 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	631	MW785819.1
Rhizopus arrhizus strain GEA 616 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1151	1151	100%	100	629	MW345817.1
Rhizopus oryzae strain UWFP 846 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1151	1151	100%	100	627	AY213685.1
Rhizopus arrhizus strain SFR-7 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1146	1146	100%	99.84	654	MT540020.1
Rhizopus oryzae UICC 28 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	1146	1146	100%	99.84	655	LC514310.1
Rhizopus oryzae UICC 11 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	1146	1146	100%	99.84	655	LC514302.1

Acc. Len =Accession Length, Per. Ident = percent identity.

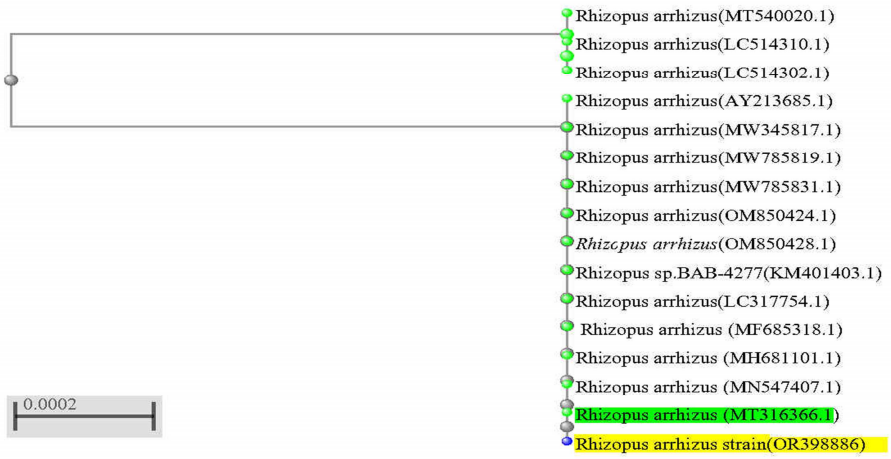

Figure 4: Neighbor-joining tree based on rDNA sequences tree showing the relationship between the isolated fungal strain *Rhizopus arrhizus* (Accession no.OR398886 with yellow high light) and aligned with closely related strains accessed from the GenBank NCBI database the genus *Rhizopus arrhizus* strain DTO400-C5 (Accession: MT316366.1 with Green high light)
Bar=0.0002 substitutions per site

3.4. Identification of fungal chitosan polymer using ^{1}H nuclear magnetic resonance (^{1}H NMR) assay

The current analysis demonstrates that the spectrum data for chitosan completely match the predicted chemical structure. Results in Fig. (5) and Table (3) showed that the peaks from 5.52 to 3.86 ppm correspond to the seven hydrogens of the glucose skeleton of chitosan, and their total integral is designated as 7.00. Peaks from 5.66 to 5.86 ppm and the peak at 1.43 theoretically correlate with 0.36 hydrogens and 0.04 hydrogens. Results also indicated that the chemical shifts at 5.52, 4.55, 4.33, and 3.86 ppm were attributed to H1, H3, H5, H6, and H2 & H4 of fungal chitosan. According to [38], Seven hydrogens on the glucose skeleton of chitosan and the peak of the hydrogen in the cyclohexyl group's -CH- overlapped, and the total integral of these eight hydrogens is taken to be 8.00. Whereas [39] reported that the chemical shifts at 5.5, 3.6–3.9, and 3.0 ppm were attributed to [H1], [H3]–[H6], and [H2] of chitosan.

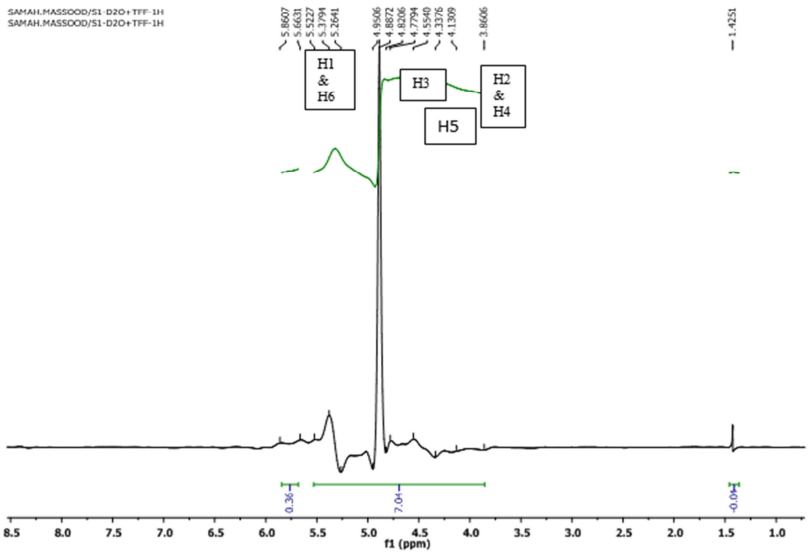

Figure 5: ^{1}H NMR spectrum of chitosan (500 MHz, D2O/TFF)

Table (3): Chemical shifts of proton in glucose skeleton of the produced fungal chitosan polymer

Proton atom ^{1}H NMR	Chemical shift (ppm)	
	Chitosan assignment result cited from Wang *et al.* (2016)	The produced Fungal chitosan
H_1	5.22	5.52
H_2	4.08	3.86
H_3	4.35	4.55
H_4	4.08	3.86
H_5	4.26	4.33
H_6	522 - 4.72	5.52 – 4.95

3.5. Evaluation of the antibacterial activity of *Rhizopus arrhizus* RL chitosan polymer

The effect of *Rhizopus arrhizus* RLand tested antibioticsof Amoxicillin/ Clavulanic acid (positive control) as antibacterial activity were studied in this experiment using agar well diffusion against pathogenic Gram-positive and -negative bacterial strains of *E. coli* O157H7,*Staph. aureus* As4,*S. typhimurium* As3, *S. shag* As4,*B. cereus* DSMZ345, and *L. monocytogenes* DSMZ 423. Data represented in Fig. (6 a) showed that all the tested pathogenic bacteria were sensitive to fungal chitosan polymer, which the growth inhibited after 24 h incubation periods at 37°C and the inhibition zone diameter ranged from 15 ± 0.70 mm to 24 ± 0.70 mm. As well as, these strains were inhibited with the tested antibiotics, ranging from 20 ± 1.41 mm to 31 ± 0.70 mm. In the case of G^{+ve} pathogenic bacteria, the fungal chitosan polymer or standard antibiotic gave a high difference significant IZD in order as follows: 22±1.76 or 31±0.70> 21±1.41 or 26±0.70> 15±0.70 or 25±1.41 mm against *Staph aureus* As4, *L.monocytogenes* DSMZ 423 and *B. cerus* DSMZ345, respectively. While the G^{-ve} pathogenic bacteria, the fungal chitosan polymer or standard antibiotic gave a high difference significant IZD in order as follows: 24 ±0.70 or 28 ±1.06> 19±1.41 or 24±1.76> 18±1.41 or 20±1.41 mm against *S. typhimurium* As3, *S. shag* As4 and *E. coli* O157H7, respectively.The percentage of antibacterial activity was calculated according to IZD of fungal polymer to the tested antibiotic, which ranged from 60.0 to 90.0%, as presented in Fig. (6 b). Results indicated that the highest activity percentage was 90.0% against *E. coli* O157H7 followed by *S. typhimurium* As3with 85.7% and *L. monocytogenes* DSMZ 423 at 80.7%. While the lowest inhibition activity was 60.0% toward *B. cereus* DSMZ345. Furthermore, the *Rhizopus arrhizus* RL chitosan polymer was more efficient against Gram-negative microorganisms than Gram-positive microorganisms. This might be because Gram-negative microorganisms' layers of peptidoglycan are thinner than those of Gram-positive microorganisms [40]. At the same time, [41] revealed how adding chitosan to enzymes produced by bacteria and nucleotides caused disruption of the cell structure of *E. coli and Staph. aureus.* The capacity of chitosan to change the concentration of calcium on cell walls. According to [42,43], gram-negative microorganisms appear to be more sensitive to chitosan and its derivatives because their cell surfaces have a larger level of negative charge. In addition, with Gram-positive organism's pathogenesis microorganisms [44] revealed that Gram-negative pathogenesis microorganisms were more responsive to probiotic strains. Probiotic colonies with inhibition zone diameters ranging from 10 to 20 mm were more effective in inhibiting *E. coli* O15H7, *S. typhimirium* As3, and *Shigella shigae* As2 than other bacteria.

3.6. Determination of MIC by well diffusion agar plate

The MIC value of some fungal polymer was used against the tested pathogenic bacterial strains. Four sequential concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/ml were used with a two-fold dilution fungal chitosan polymer. Results in Table (4) showed the superiority of fungal chitosan polymer, where the MIC value was 12.5mg/mL for *S. typhimurium* As3, and 25 mg/ml for other the tested pathogenic bacterial strains of *S. shag* As4 *E.coli* O157H7, *B. cereus* DSMZ345, *L. monocytogenes* DSMZ423 and *Staph. aureus* As4. Results also showed that the antibacterial spectrum activity of tested fungal chitosan polymer against 6 pathogenic strains at concentrations ranging from 10 to 2.5 mg/mL was 100%, and at a concentration of 1.25 mg/ml achieved activity 16.6 %.

These results agree with [45], who reported Antibacterial action against multidrug-resistant pathogenic microbes, such as *Enterococcus faecalis*, *E. coli*, *Staph. aureus*, and *Klebsiella pneumonia*. The antimicrobial activity analysis of low and high-molecular-weight chitosan nanoparticles CSNPs have shown effectiveness against *E. coli* O15:H7 [46]. Additionally, [47] observed that the MIC values for *P. aeruginosa* Ps9 ranged from 0.08 to 0.16 mg/mL and were 0.32 mg/mL for *Staph. aureus* St3, *E. coli* Ec3, and *S. typ*hi Sa1 respectively. The minimum inhibitory concentration (MIC) of CuNPs existed 50

µg/mL. According to [48], the minimum lethal concentrations (MLC) for *Staph. aureus* ATCC 5638 and *Aspergillus flavus* ATCC 9643 were 50 and 75 g/mL, respectively. Furthermore, [44], reported the tested fermented soymilk cultures' MIC values against *E. coli*, *Shigella shigae* As2, *S. typhi*, *Staph. aureus*, *B. cereus*, and *L. monocytogenes* ranged between 3.72 and 2.11CFU/mL (0.25 and 0.125 dilutions).

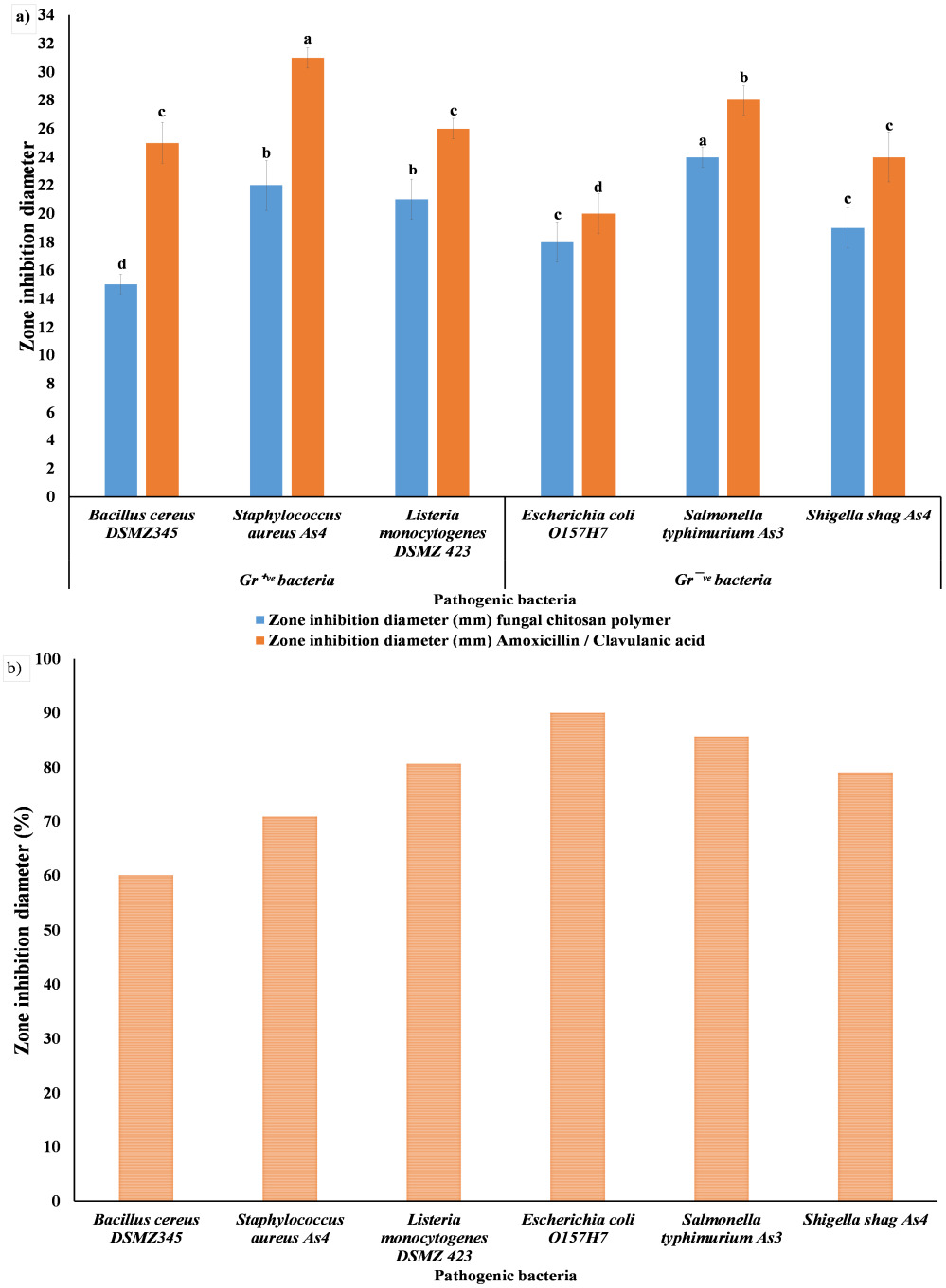

Figure 6: (a) Diameter inhibition zone (mm) and (b) inhibition activity of *Rhizopus arrhizus* RL chitosan polymer and antibiotics (Amoxicillin / Clavulanic acid) against Gram^{+ve} and Gram-ve pathogenic bacteria; a, b Means values with small letters above the column followed by different letters are significantly different at $p < 0.05$ level. Bar indicated to± standard division (SD)

Table 4: Minimum inhibitory concentration (MIC) of *Rhizopus arrhizus* RL chitosan polymer against pathogenic bacteria

Two-Fold dilutions	Concs. (mg/mL)	Pathogenic bacterial strains						Spectrum activity (%)
		B. cereus DSMZ345	*Staph. aureus* As4	*L. monocytogenes* DSMZ423	*E. coli* O157H7	*S. typhimurium* As3	*S. shag* As4	
1.0	10.00	-	-	-	-	-	-	(6/6) 100%
1/2	5.00	-	-	-	-	-	-	(6/6) 100%
1/4	2.50	-	-	-	-	-	-	(6/6) 100 %
1/8	1.25	+	+	+	+	-	+	(1/6) 16.6%
1/16	0.625	+	+	+	+	+	+	(0/6) 0%

Concs., Concentrations; -, no growth (inhibited with agent); +, growth; ND, not detected

3.7. Cytotoxicity activity of the chitosan polymer produced *R. arrhizus* RL strain on the Vero cell

Vero is a basic CCL-81 kidney cell line. Cells those are adhesive and epithelial. This study assessed the cytotoxicity activity of chitosan polymer made from the *R. arrhizus* RL strain against the Vero cell line using the MTT technique at six doses. The Vero cell maintained a percentage of viable cells that varied from 75.8% to 99.6% after being exposed to a fungal polymer at a concentration of up to 250 g/mL for 24 hours, according to the findings given in Fig. (7). The Vero cell maintained a percentage of viable cells that varied from 75.8% to 99.6% after being exposed to a fungal polymer at a concentration of up to 250 g/mL for 24 hours, according to the findings given in Fig. (7). Therefore, the polymer did not exhibit any cytotoxicity in the cell line at concentrations of 31.25, 62.50, and 125 g/mL, with an inhibition percentage ranging from 0.4 to 1.6% even though cytotoxicity was not seen because more than 50% of the cells were still alive even though the cell viability dropped to 75.8% and was reduced to 24.2% at 250 g/L. While the cell viability was significantly decreased to 2.5% and the toxicity increased to 79.5% at high fungal polymer concentrations of 500 and 1000 g/mL, respectively, the cell viability was reduced to 22.3% (with inhibition of 77.7%).

To calculate the half-maximal (50%) inhibitory concentration (IC_{50}), GraphPad Prism version 5 was used. Vero cells reported a high R^2 of 0.96 and an IC_{50} value of 367.49±7.16 µg/ml (Fig. 7). According to [47] calculations, the IC_{50} values represent 50% cell viability inhibition.

Using an inverted phase-contrast microscope with a magnification of 100x, morphological modifications of the cell line were studied following treatment with different concentrations of fungal chitosan polymer (range from 31.25 to 1000 g/ml) (Fig. 8). The Vero cells survived and had typical adherent cells when exposed to a fungal chitosan polymer of up to 250 g/ml. While the cell shape changed, especially at higher concentrations of fungal polymer (500 and 1000 g/ml), it appears apoptotic cells (cell shrinkage), cell debris, as well as much fewer cells overall, indicate cell death in comparison to control.

[49] employed Vero cells in cytotoxicity testing, which is a common practice for international studies using biomaterials with fibroblast-like characteristics. As shown by the extract results of cytotoxicity tests with polymeric hydrogel blends, Vero cells showed a morphological pattern that was strikingly similar to the growth pattern of the cells on the culture plate, forming a confluent monolayer of cells to predominantly elongated morphology, indicating no cytotoxicity of biomaterials. Furthermore, [50] discovered that chitosan-Ag nanoparticles displayed no cytotoxicity against the Vero cell line at every concentration examined. Similarly, L-929 fibroblast cells were not harmed by 312.5 g/ml of chitosan-Ag nanoparticles. Chitosan and Gantrez® nanoparticle concentrations up to 1000 g/mL had no effect on cells' metabolic activities or viability.However, cellular survival and metabolic activities were decreased when poly L-lactide-co-glycolide (PLG) microparticles were applied at 1000 g/ml. [51] revealed that all nanoparticles and microparticles were determined to be non-cytotoxic at doses below 1000 g/ml.

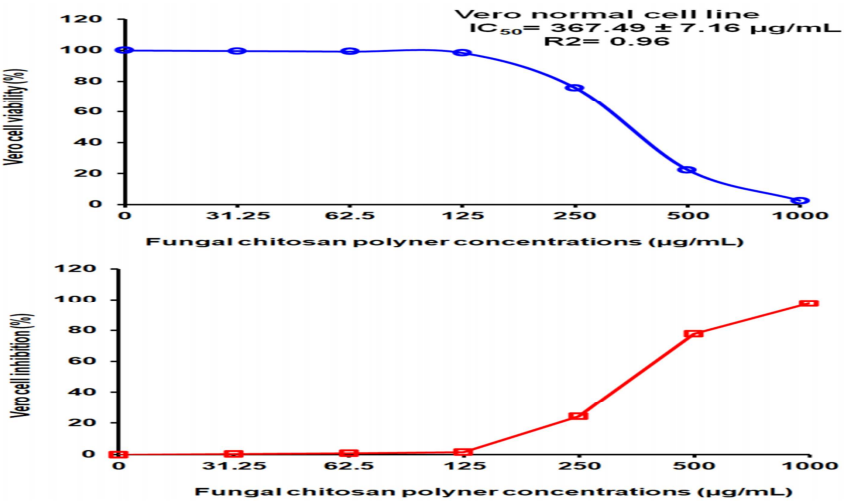

Figure7: Vero normal cell line viability and inhibition percentage after treatment with various concentrations of fungal chitosan polymer (ranged from 31.25 to 1000 µg/ml

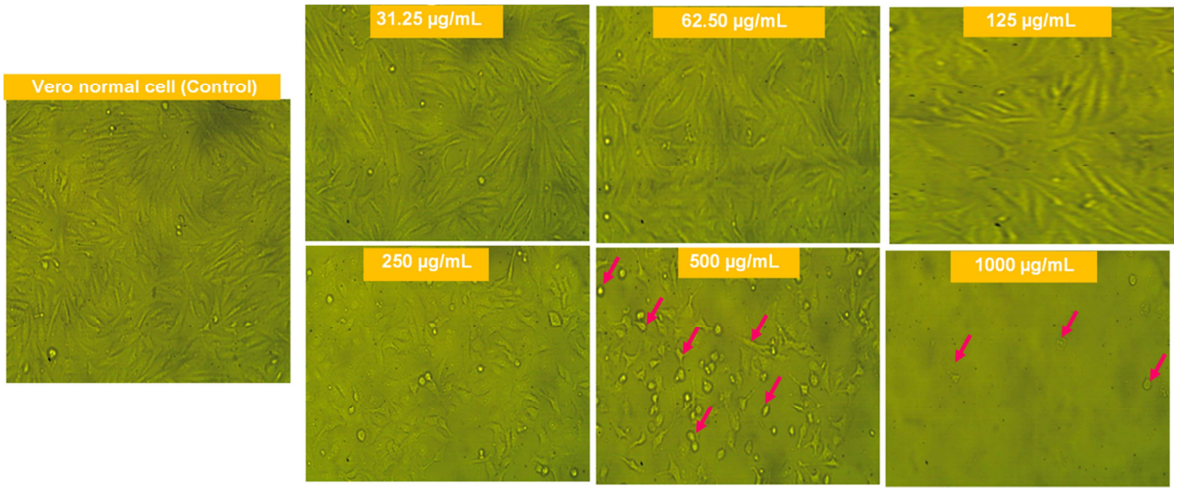

Figure 8: Vero normal cell line morphological changes of the cell line after treatment with various concentrations of fungal chitosan polymer (ranging from 31.25 to 1000 µg/mL), photographed with an inverted phase-contrast microscope at a magnification of 100×
The pink arrows on the image indicate apoptotic cells (cell shrinkage), cell debris, and major decreases in cell number. An ethanol mixture extract included Chamomile, Sage, and Cinnamon extracts.

Conclusions

The *Rhizopus arrhizus* RL strain was given the most chitin and chitosan polymer of the ten fungal isolates isolated from the soil. The fungal chitosan polymer chemical structure was confirmed using ^{1}H NMR. The fungal polymer had antibacterial activity with MIC values ranging from 2.5 to 1.25 mg/ml, this natural polymer inhibited Gram-positive and -negative foodborne bacteria and multidrug resistance, such as *Staph. aureus* As4. In addition, the tested polymer didn't have cytotoxicity. Therefore, the fungal chitosan polymer can be converted into polymer film and used to coat foods to increase shelf life and preservation**.**

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