Microscopic and Molecular Diagnosis of *Aspergillus* Fungus Associated with Local Wheat Grains

Ruaa Kamel Mahmoud[1*] and Maryem Akbal Hasoon[2]

[1]College of Agriculture, Al-Qasim, Green University, Babylon, Iraq
[2]Al-Mussaib Technical College, Al-Furat AL-Awsat Technical University, Babylon, Iraq.
*Corresponding author's email:
ruisanno2010@gmail.com
maryam.akbal.cmh123@atu.edu.iq

Abstract

In this study, fungus associated with wheat grains were isolated and identified microscopically and molecularly by evaluating genetic variations within ribosomal sequences to determine the fungal isolates' biodiversity pattern. The results we obtained showed the precise identities of the amplified samples. Sequence assays showed that sample Y4 was 99% identical to *Aspergillus terreus*. Evolutionary analyses also validated its placement within the respective clades, with the Y4 isolate being thought to have originated in India.

Keywords: *Aspergillus terreus*, *Triticum aestivus*, Wheat Grains

Introduction

Wheat *(Triticum aestivum* L.) is the most widely produced crop in the world and Iraq, with the highest production volume and economic return. It is a staple food for about 1.5 billion people in 40 nations, or 35% of the world's population. Global wheat production fell dramatically in 2022, reaching 770.3 million tons, 1.0% less than in 2021[3]. Wheat is the world's most frequently cultivated and traded grain, and it serves as the primary energy source for the human body. It contains proteins, minerals, vitamins, and lipids, and grains provide 70% of the body's caloric requirements [10].

Depending on the temperature and humidity conditions in the storage environment, grains are impacted by a various fungi,These fungi can cause significant economic losses by Lowering agricultural output causes seedling death before and after germination and affects embryo growth, resulting in lower germination rates [6]. Certain fungal species, particularly those from the genera *Aspergillus*, *Penicillium*, and *Fusarium*, are among the most harmful food pollutants because they produce mycotoxins such as *aflatoxins*, *ochratoxins*, and *fumonisins*, which are carcinogenic to both people and animals. These poisons remain in contaminated crops even after thermal processing and can infiltrate the food chain, disrupting the immune system and causing irreversible DNA damage [12]. As a result, the experiment aimed to separate fungi associated with wheat grains and identify them morphologically and molecularly, with a focus on the most common and widespread fungal isolate.

Material and Methods

Isolation and identification of fungi associated with local wheat grains:

Fungi associated with local wheat grains were isolated utilizing Petri dishes and PDA (Potato Dextrose Agar) media. Ten grains were placed in each dish directly into the PDA medium, arrayed in a circular pattern 1 cm from the dish's edge, with three replicates of each. The plates were incubated at a temperature of 25 ±

2 °C. After five days, the fungi were identified and their frequency and occurrence percentages calculated. Each fungal isolate was purified on a new medium to verify identification based on cultural and physical traits using recognized taxonomic keys [2] and [7]. The percentage frequency of the fungus was determined using the following formula:

$$\textbf{Fungal Frequency}\% = \frac{\textbf{Number of fungus isolates in samples}}{\textbf{Total number of isolates in samples}} * 100$$

The occurrence percentage of fungal colonies growing in the plates for each fungus was calculated utilizing the formula:

$$\textbf{Fungal Occurrence } \% = \frac{\textbf{Number of samples in which the fungus appeared}}{\textbf{Total number of samples}} * 100$$

Molecular identification of aspergillus strain utilizing PCR technique:

A pathogenic isolate of the *Aspergillus genus* (spp.) was identified and isolated from local wheat grains. The fungus was cultured on a Petri dish with PDA medium and 100 mg L^{-1} of antibiotic Chloramphenicol for seven days at 28°C ± 2. After this time, the aerial mycelium was collected with a sterile small brush to confirm that no agar was present. The mycelium was then placed into 1.5 mL Eppendorf tubes for fungal isolation. The examination was conducted out in the College of Pharmacy laboratory at the University of Babylon.

DNA extraction and purification:

The method used was based on Munoz-Cadavid et al. [5], with the inclusion of proteinase K enzyme, as described by [4] and [8]. The following measures were taken:

1- The fungal mycelium was transferred from the Petri plate to an Eppendorf tube with 293 µL of EDTA and crushed with wooden sticks.
2- A micropipette was used to break the fungal cells by repeatedly pipetting (suction and expulsion) until the biomass stayed floating and did not float on the surface or settle at the bottom of the tube.
3- Each tube received 7.5 µL of Proteinase K (20 mg mL^{-1}).
4- The tubes were vigorously combined with a vortex mixer several times.
5- The tubes were incubated at 56°C for 60 minutes, allowing the Proteinase K enzyme to degrade cell membranes and walls.
6- The samples were centrifuged at 14,000 rpm for two minutes.
7- 300 µL of Nuclei Lysis Solution was added to the tubes and mixed gently with the vortex mixer.
8- 100 µL of Protein Precipitation Solution was added to the tubes and gently mixed with a vortex mixer.
9- The samples were stored on ice for 5 minutes before centrifugation at 14,000 rpm for 3 minutes.
10- To precipitate the DNA, the supernatant was transferred to clean, sterile Eppendorf tubes containing 300 µL of 50% isopropanol at room temperature.
11- The tubes were gently stirred to examine the DNA pellet, then centrifuged at 14,000 rpm for 2 minutes. To wash the precipitated DNA, the supernatant was taken out from each tube, and 300 µL of 70% ethanol was added. The tubes were gently inverted many times.
12- The contents were centrifuged at 12,000 rpm for 2 minutes at room temperature before extracting the ethanol with a micropipette.
13- The tubes were air-dried by turning them upside down on absorbent paper for 30 minutes to allow the ethanol to drain totally.

14- Rehydrate DNA solution (50 µL) was added.
15- To purify the DNA from RNA, 1.5 µL of RNase Solution was added to the tubes, and the mixture was thoroughly vortexed for a few seconds. The samples were then incubated at 37°C for 15 minutes to induce RNA breakdown.
16- The tubes were heated at 65°C for 60 minutes to rehydrate the DNA and dissolve the DNA pellet at the bottom of each.
17- The samples were stored at -20°C until additional amplification processes were completed.

The concentration and purity of the obtained DNA were evaluated utilizing a Nanodrop equipment (Scandrop, Biometra, Germany). Sample concentrations were between 40-60 ng μL^{-1}, with DNA purity ratios (280/260 and 230/260) ranging from 1.7-1.8. Samples having purity ratios of less than 1.7 were reextracted.

Analysis of similarity percentage between aspergillus isolate and global strains:

The polymerase chain reaction (PCR) was executed using a kit provided by Macrogen Inc. The PCR reaction was carried out in a total volume of 25 µL, employing 2 µL of DNA and 1 µL of each primer: forward primer ITSf 5'-TCCGTAGGTGAACCTGCGG-3' and reverse primer ITSR 5'-TCCTCCGCTTATTGATATGC-3' [11]. All reagents were added to the PCR tube in the order specified in (Table 1). The fungal DNA was amplified under the following PCR conditions:

Table 1. PCR reaction materials

Chemical	Volume (µL)
PCR master mix solution	10
Forward primer	1
Reverse primer	1
Nuclease-free water	10.5
DNA	2
MgCl	0.5
Final reaction volume	25

The samples were centrifuged in a PCR tube before being transferred to the thermal cycler. The reaction was carried out by programming the machine employing the following procedure:

Step 1: Initial Denaturation: One cycle of 5 minutes at 94°C.

Step 2: Three temperature steps repeated 35 times: Denaturation time: 30 seconds at 94°C.

Annealing time is 30 seconds at 58°C, which allows the primers to bind to the DNA template.

Extend for 40 seconds at 72°C.

Step 3: Final Extension: One cycle of 5 minutes at 72°C.

Step 4: Cool. The reaction was cooled to 4 °C.

Electrophoresis with Agarose Gel:

Electrophoresis was carried out with agarose gel (Agarose gel) after dissolving 1 gram of agarose powder in 100 mL of 1% TBE buffer solution. The mixture was properly combined and microwaved until it reached boiling point and all components had dissolved.

After cooling to 50°C, add 5 µL of the fluorescent dye Ethidium Bromide to the gel and mix thoroughly. The agarose gel casting tray was constructed with a comb at one end to produce wells in the gel. The agarose gel solution was put into the electrophoresis tray, which held the comb, and allowed it to harden at room temperature for 15 minutes. The comb was gently removed afterwards.

Next, the TBE buffer solution was added to the electrophoresis tank, covering the agarose gel to a height of about 1 cm. Five µL of a DNA ladder marker was added to the left hole to determine the amplified DNA's size. After that, five µL of amplified

The electrophoresis tank lid was covered, and the electrophoresis apparatus was connected to the electrical supply. For one hour, the power supply was set to 100 mA at 70 V. Following electrophoresis, the gel layer containing the DNA products was examined with a UV light source, and images were acquired [9] and [1].

Analysis of the nucleotide sequence of DNA from fungal isolates:

The PCR-amplified products of the fungal isolates were sent to Macrogen Inc. (Geumchen, Seoul, South Korea) for nucleotide sequencing. The primers used for amplification were utilized to determine the nucleotide sequence. The sequences were processed using DNASTAR version 7.1 (Madison, WI, USA), and the results were compared to sequences recorded in the National Center for Biotechnology Information (NCBI) for globally known fungal isolates.

Results and Discussion

Isolation and identification of fungi associated with local wheat grains:

The results given in table (2) show the identification of five fungal species isolated from local wheat grains using morphological characterization of the samples analyzed (Figure 1). The results showed that *Aspergillus spp*. was the most common and abundant fungus, with a frequency of 50.617% and a 100% appearance rate when compared to other fungi (Figure 2).

Table 2. Fungi isolated from local wheat grains:

Fungi	Frequency (%)	Appearance (%)
Aspergillus	50.617	100
Alternaria	20.987	26
Fusarium	13.345	37
Penicillium	11.345	15
Bipolaris	3.706	11

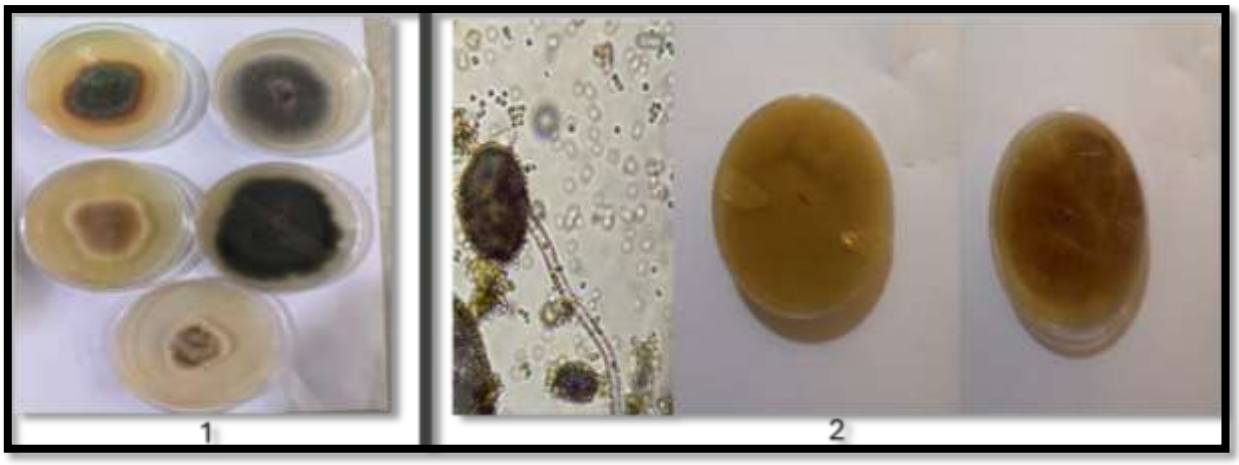

Figure 1. shows the types of fungi isolated from local wheat grains, while figure (2) illustrates the colony of *Aspergillus fungus*.

Molecular diagnosis of the *Aspergillus* strain utilizing the PCR method:

The molecular diagnostic results for the *Aspergillus spp*. isolate, through DNA sequencing with the ITS4-ITS1 primers within the rRNA gene region and electrophoresis on an agarose gel (Figure 3), revealed clear bands for the fungal isolate, with the molecular size estimated to be approximately 542 base pairs (\bp) (Figure 4).

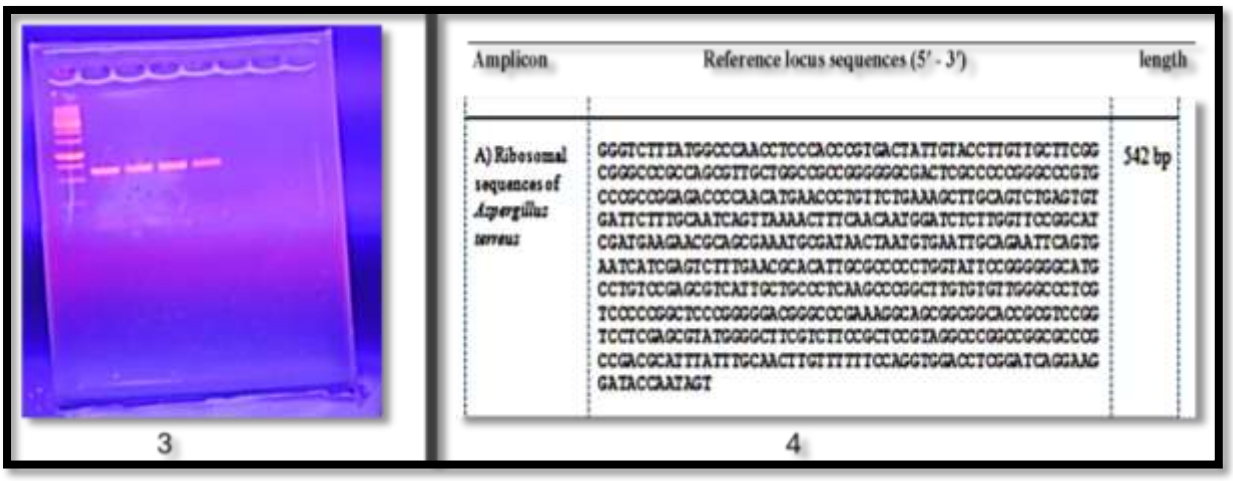

Figure (3) shows electrophoresis of an *Aspergillus spp*. isolate on an agarose gel for PCR product using ITS1 and ITS4 primers, with marker bands highlighted, whereas figure (4) shows DNA fragment position and length for an *Aspergillus* isolate.

Comparative Analysis of *Bipolaris* spp. Isolates and Global Strains:

The recommended primers effectively amplified the *Aspergillus spp*. isolate and identified its nucleotide sequence. The NCBI BLASTn analysis revealed a significant degree of similarity between the currently analyzed samples and worldwide *Aspergillus spp*. sequences when the DNA sequences were compared to those in GenBank (GenBank accession number: MH297432.1). The identified fungal isolate was entered into the NCBI database with the accession number PP373784.

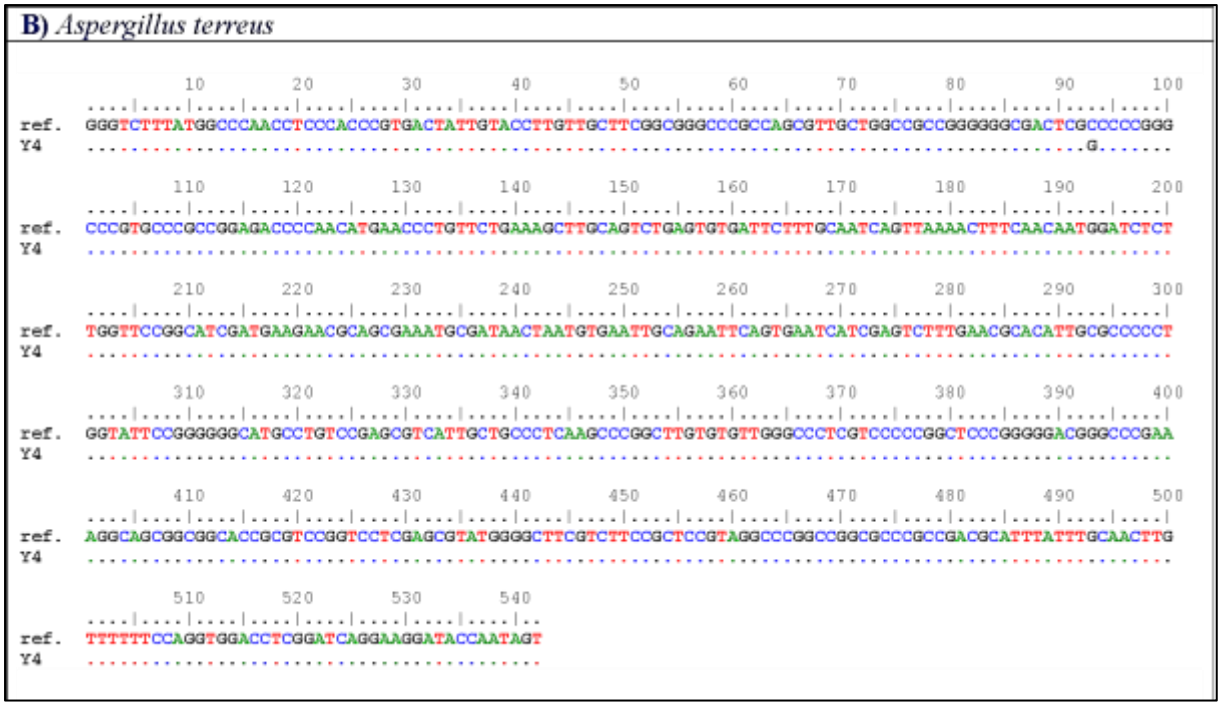

Figure (5) shows a comparison of the fungal isolate's DNA sequence to its reference sequence from the NCBI GenBank DNA database.

These DNA sequence differences were identified in the samples examined as compared to the database sequences. The color-coded sequence of the analyzed material was validated and documented, with graphs illustrating sequence positions relative to PCR amplification sites.

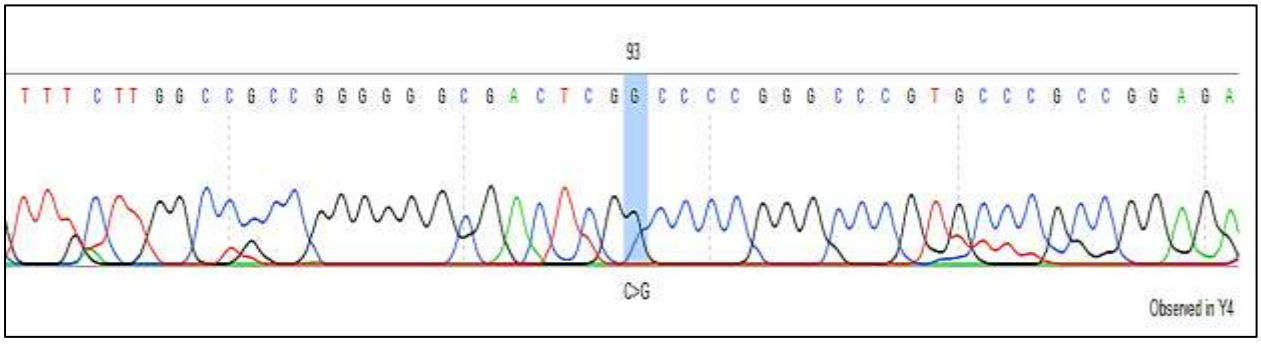

Figure (6) shows a color-coded variation map of the Aspergillus fungal isolates examined.

A comprehensive phylogenetic tree was generated for the isolated fungal samples, as well as additional DNA sequences deposited in GenBank. According to the genomic sequences analyzed, this tree contained only one genus, *Aspergillus*. *Aspergillus* rRNA sequences were compared to numerous surrounding genetic groupings, demonstrating that this genus has a different diversity range in terms of the rRNA sequences analyzed (Figures: 4,5,6,7 and 8). These adjacent groups included *A. alabamensis* and *A. hortae*. However, all species, including *A. alabamensis* and *A. hortae*, were classified as external species within the studied fungal genus. The result revealed that the closest relative sequences for the studied sample belonged to *A. terreus*. Remarkably, minimal branch distances (tree size 0.01) were found, indicating that the fungi are extremely similar.

When compared to the non-redundant GenBank database, the sequence was correct 99% of the time (Figure 6).

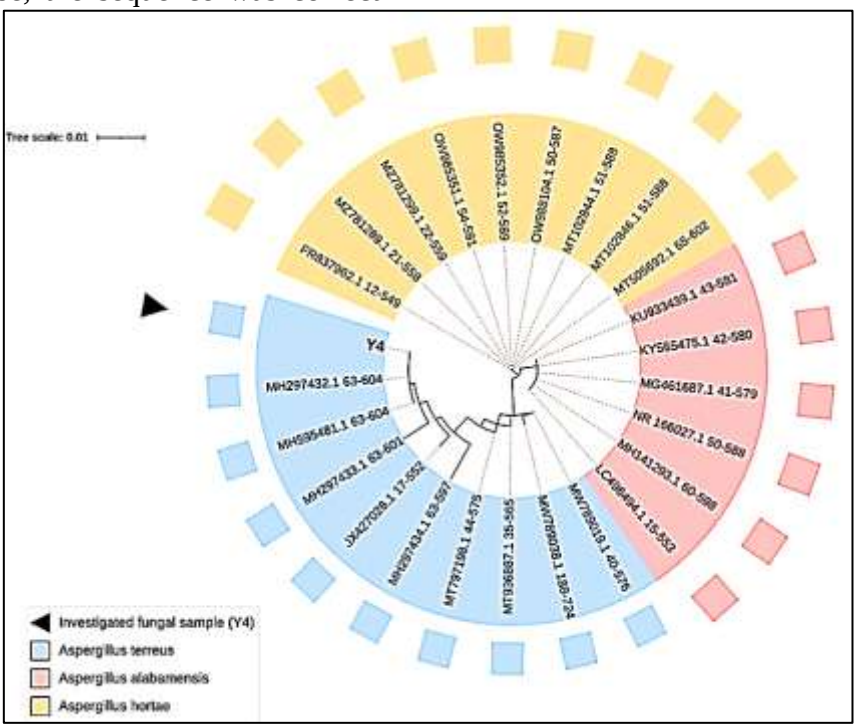

Figure (7) shows a comprehensive phylogenetic tree, which partially covers the ITS sequences of the *Aspergillus terreus* fungus isolated in this study.

Conclusion

In conclusion, molecular analysis of *Aspergillus spp*. isolates using PCR amplification with ITS4-ITS1 primers and electrophoresis revealed distinct bands with a molecular size of around 542 base pairs. The NCBI BLASTn analysis revealed strong sequence similarity with global *Aspergillus spp*. strains, and the fungal isolate was entered into the NCBI database. A complete phylogenetic tree based on rRNA sequences revealed that the isolates belonged mainly to the *Aspergillus terreus* species, with low genetic distance observed between the examined isolates, indicating a high level of closeness. A comparison with the GenBank database revealed a 99% similarity, supporting the isolates' identity and genetic links.

Acknowledgment:

I would want to offer my heartfelt appreciation to everyone who helped and contributed to this study. Thank you to the staff members and researchers at the NCBI database for their wonderful resources and assistance.

References

[1] **Al-azawy, A. F. (2011).** A rapid, non-enzymatic method for genomic DNA extraction from whole blood and mammalian tissues. *Research Opinions in Animal and Veterinary Sciences*, 1(5), 279-283.

[2] **Ellis, M.B.** (1971). *Dematiaceous Hyphomycetes*. Commonwealth Mycological Institute, KEW, England.

[3] **Horn, B., Ferreira, C., and Kalantari, Z.** (2022). Links between food trade, climate change and food security in developed countries: A case study of Sweden. *Ambio*, 1-12.

[4] **Mirhendi, H., Makimura, K., Zomorodian, K., Maeda, N., Ohshima, T., and Yamaguchi, H.** (2005). Differentiation of *Candida albicans* and *Candida dubliniensis* using a single-enzyme PCR-RFLP method. *Japanese Journal of Infectious Diseases*, 58(4), 235.

[5] **Muñoz-Cadavid, C., Rudd, S., Zaki, S. R., Patel, M., Moser, S. A., Brandt, M. E., and Gómez, B. L. (2010)**. Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: Comparison of five tissue DNA extraction methods using panfungal PCR. *Journal of Clinical Microbiology*, 48(6), 2147-2153.

[6] **Niaz, I., and Dawar, S**. **(2009).** Detection of seed-borne mycoflora in maize (*Zea mays* L.). *Pak. J. Bot.*, 41(1), 443-451.

[7] **Pitt, J.I., and Hocking, A.D. (1997).** *Fungi and Food Spoilage*. Blacki Academic and Professional, 593 pp.

[8] **Sambrook, J., and Russell, D. W. (2001)**. *Molecular Cloning: Ch. 15. Expression of cloned genes in Escherichia coli* (Vol. 3). Cold Spring Harbor Laboratory Press.

[9] **Sambrook, J., Fritschi, E. F., and Maniatis, T. (1989).** *Molecular Cloning, a Laboratory Manual* (3rd ed.). Cold Spring Harbor Laboratory Press, New York.

[10] **Shewry, P. R., and Hey, S. J. (2015)**. The contribution of wheat to human diet and health. *Food and Energy Security*, 4(3), 178-202.

[11] **White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. (1990).** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, 18(1), 315-322.

[12] **Yasuki, and Kubota. (2006)**. Microorganism control using ultraviolet irradiation. *In Summary of 57th JsAM Annual Meeting*.