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Analyzing the Fungal Biodeterioration of Oil Seeds: Isolation, Characterization, and DNA Extraction

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Abstract: *In the study of isolation and characterization of fungi associated with oil seeds was done. The peanut samples were collected from local areas. The peanut samples were kept in moisture for one week for the growth of fungus. Two varieties of peanuts were taken one from the market and one direct from farm. Peanut kernels and Raw peanuts. The samples were kept at room temperature and in incubator both for 1 week. After 1 week growth of fungi was observed. Different media were prepared for the isolation of fungi. The media that were prepared for isolation were potato dextrose agar and czpeck dox agar. The fungus obtained on the peanut varieties Peanut kernels and Raw peanuts was streaked on potato dextrose and czpeck dox agar media. After few days growth was observed and lactophenol cotton blue staining was performed. The different parts of fungi like hyphae, conidiophore were observed. After observation under microscope a perfect grown fungus was taken for subculturing and after that DNA isolation was performed to observe DNA bands.*

Key Words: *fungal isolation, oilseeds, morphplogical characterization, DNA isolation.*

1. INTRODUCTION:

Fungi are widely distributed in nature. It is estimated that there are 1.5 million species of fungi on earth. Both beneficial and harmful. The fungi are spore bearing eukaryotic organisms without chlorophyll and having absorptive nutrition. These reproduce sexually as well as asexually. Primarily, these are terrestrial microorganisms, though some are present in aquatic environment also, both in marine and fresh water. Many are pathogenic to plants, animals and humans. Some fungi are present in beneficial relationship with other organisms (Mycorrhiza), lichens, etc.[1]

A fungus can be identified by observing its growth on slide culture and plate culture, and also by observing its hyphae and sporulating structures microscopically under low and high power.

Fungi grow in moist environment and are chemoautotrophs. They use organic compound as a source of energy, electron and carbon. Most fungi grow on dead organic matter by producing exoenzymes. These are saprophytes, parasitic. Fungi are usually aerobic though some are anaerobic.[2] The vegetative structure of fungus is called thallus. It varies in complexity and size ranging from unicellular yeast to multicellular moulds. The mass of inter-coiling branched, thread-like structures called hyphae are present. The mass is known as mycelium. The hyphae grow on or within the nutrient media to get nutrients that represent vegetative mycelium. Some fungi are dimorphic, i.e. at 37˚ C these grow as yeast but at 25˚ C change to mould structure. This shift is known as YM shift.[3] Fungi are classified as:

Zygomycota: Hyphae in this subclass are coenocytic. Asexual spores developed in sporangia at the tips of areal hyphae. Sexual spores are called zygospores involved in production of food like tempeh, sufu. Ex. Rhizopus, Mucor.

Ascomycota: Ascomycetes are also known as sac fungi because sexual reproduction involves formation of ascus containing ascospores. Asexual reproduction occurs through conidiospores. These have septate mycelium. Ex. Claviceps, Neurospora.

Basidiomycota: Basidiomycetes are also called club fungi. Their sexual structure is basidium which produces basidiospores. Involved in plant decomposing. Also causes plant, animal and human diseases. Toxins or hallucinogens are produced by certain mushrooms. Ex. Rusts, mushrooms, puff balls.[4]

Deuteromycota: Deuteromycetes are also called imperfect fungi. Asexual reproduction occurs by conidia. Fungi imperfecti are important as they are human pathogens. Few are industrially important and involved in antibiotic, food production. Some produce mycotoxins which are highly toxic and carcinogens. Ex. *Aspergillus*, *Penicillium*.[5]

Oil seeds or crops such as flaxseed are mainly grown for edible oil extraction. The ability of an oil seed to adjust to seasonal variations during development is an indicator of its phenological plasticity.[6]

There are several types of oil seeds: Groundnut, soybean, mustard/rapeseed, sesame, safflower, linseed, flaxseeds, etc.

2. LITERATURE REVIEW:

R Kakade. and A. Chavan. (2011). Damage to the oil seeds have been reported to be caused by fungi associated with them. Fungi like *Aspergillus niger*, *Aspergillus flavus*, *Alternaria dienthicola*, *Curvularia lunata*, *Fusarium oxisporom*, *Rhizopus stolonifera*, *Penicillium cricogenum* causes discoloration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification to oil seeds. As oil seeds are rich in oil content, which boost the vigor of pathogenic fungi resulting in biodeterioration by production of lipase. Fungi growing on stored grains, can reduce the germination rate along with loss in the quantum of carbohydrate, protein and total oil content, induces moisture content, free fatty acid content enhancing other biochemical changes of grains.

S Chaudhary, et al (2018) have found that the number and diameter of fungal discs of the test fungus had no effect on fungitoxicity, indicating that they can sustain heavy inoculums at MIC. The extract of Hedychium spicatum was discovered to have a broad fungitoxic spectrum, inhibiting the mycelial growth of 20 different fungi at its MIC.

S Groot, et al (2022) studied that peanuts are transported by ship from production regions to destinations around the world. Quality issues are frequently encountered as a result of increased free fatty acid (FFA) levels and a decline in organoleptic quality caused by lipid oxidation during transport and storage. They investigated the role of moisture (water activity, aw) in interaction with 87 days of hermetic storage in air or nitrogen gas. When stored with air, some lipid oxidation was observed at water activity levels less than 0.73. FFA levels increased when water activity exceeded 0.73, and fungi multiplied when water activity exceeded 0.80. After storage under nitrogen gas, no lipid oxidation, FFA level increase, or fungal growth were observed. Peanut storage and transport under anoxia can significantly reduce quality losses.

R Indiarto and B Rezaharsamto (2020) Storage can lead to dark discolouration and decreased peanut hardness. Chemical changes such as increased rates of respiration, off-flavour, and rancidity reduce the quality of peanuts. While the growth of Aspergillus flavus moulds on peanuts that produce aflatoxin B1 causes microbiological damage. This toxin has been classified as hazardous and carcinogenic.

3. OBJECTIVES:

This study revolves around the following four objectives

- Isolation of fungi associated with oil seeds.
- Identification and characterization of fungi.
- To study biochemical test for characterization of fungi.
- Isolate DNA from fungus of oil seeds.

4. RESEARCH METHODOLOGY:

4.1. Materials Required:

This study's materials included peanut samples (both kernels and raw peanuts), Potato Dextrose Agar (PDA) and Czapek Dox Agar for media preparation, as well as chemicals such as sucrose, sodium nitrate, dipotassium phosphate, magnesium sulphate, potassium chloride, and ferrous sulphate. Petri plates, conical flasks, beakers, test tubes, pipettes, spreaders, needles, measuring cylinders, and culture tubes were all necessary pieces of laboratory equipment. An autoclave, laminar air flow, incubator, and pan balance were used to sterilise and culture the specimens. Chemicals such as mercurochrome for sample sterilisation, lactophenol cotton blue for staining fungal structures, and CTAB buffer, chloroform, and isopropanol for DNA extraction were all required. Finally, spectrometers and microscopes were required for DNA analysis and microscopic examination of fungal structures.

4.2. Experimental situate and Sample collection:

The experiment was conduct in Institute of Biotechnology MGM University, Chh. Sambhajinagar. The location provided access to necessary laboratory facilities and equipment essential for conducting microbiological studies, plant extract preparations, and bioactivity assessments. The sample was collected from the market and sterilized with mercuric chloride. Oil seeds, specifically peanuts, were collected from the local markets. The peanuts, *Peanut kernels* were used for the experiment.

4.3. Media Preparation and Sterilization:

Two types of media were prepared to facilitate fungal growth: Potato Dextrose Agar (PDA) and Czapek Dox Agar.

- **Potato Dextrose Agar (PDA)**: This medium was made by boiling potatoes to extract their nutrients, and then adding dextrose (a sugar) to support fungal growth.
- **Czapek Dox Agar**: This medium was composed of sucrose, sodium nitrate, dipotassium phosphate, magnesium sulphate, potassium chloride, and ferrous sulphate to support the growth of fungi.

The media prepared were sterilized in autoclave at 121 ℃ at 15 psi for 20 minutes to ensure that they were free of any contaminants.

4.4. Serial dilution:

After the peanuts were allowed to grow fungal colonies, the samples were crushed into fine particles. A series of dilutions were made, ranging from 10^1 to 10^7, to reduce the concentration of fungal spores and ensure that individual colonies could be isolated for study.

4.5. Preparation of Plates and Incubation:

Once the media cooled to room temperature, it was poured into sterilized Petri plates. The Petri plates were also autoclaved to maintain sterility. The diluted fungal samples were streaked onto the surface of the agar plates using sterile techniques to avoid cross-contamination. The streaked plates were incubated at 25°C for a period of 4-6 days. This temperature range was chosen because it is ideal for promoting the growth of common environmental fungi.

4.6. Microscopic observation:

After incubation, visible fungal colonies appeared on the agar plates. These colonies were carefully sampled and subjected to lactophenol cotton blue staining, to highlight the structure of fungi. Lactophenol acts as a mounting fluid, while cotton blue stains the fungal cell walls. The stained fungal samples were observed under a microscope using a 40X objective lens to identify features like hyphae, conidiophores, and spores, which are essential for fungal identification.

4.7. Subculturing:

To obtain pure fungal isolates, individual colonies were subcultured onto fresh PDA and Czapek Dox Agar plates. This step ensures that only a single fungal species grows on the plate, allowing for a more accurate study. Additionally, some fungal colonies were inoculated into Potato Dextrose Broth (a liquid medium) to support rapid growth and preparation for further studies. These plates and broth cultures were incubated again under similar conditions to encourage further fungal growth.

4.8. DNA Isolation:

The DNA isolation process involved several key steps. First, in the cell lysis step, the fungal mycelia were dried and crushed in 600 µl of prewarmed CTAB buffer, which helped break down the cell walls, and the mixture was incubated at 55°C for 3-4 hours to fully release the DNA. In the centrifugation step, the lysate was spun at 10,000 RPM for 10 minutes, separating the DNA-containing supernatant from the cell debris. In the organic extraction step, 600 µl of chloroform with isopropanol in ratio 24:1 was added to the supernatant, and the mixture was vortexed for 5 minutes to remove proteins and other contaminants. Following another round of centrifugation, the supernatant was transferred to a new tube. During the DNA precipitation step, pre-chilled isopropanol was added to the supernatant and the sample was kept at -50°C for 15 minutes, allowing the DNA to precipitate. After this, a final centrifugation step at 10,000-16,000 RPM for 10 minutes was performed, and the DNA pellet was washed with 70% ethanol in the ethanol wash step to remove any remaining impurities. After drying the pellet overnight in the drying step, it was resuspended in 25 µl of nuclease-free water during the reconstitution step. Finally, the DNA quality was assessed using gel electrophoresis and a spectrometer to observe the DNA bands

5. RESULT:

The study successfully isolated and characterized fungi from peanut kernels and raw peanuts using both morphological and molecular techniques. After incubation on Potato Dextrose Agar (PDA) and Czapek Dox Agar, fungal growth was observed on all the plates, with *Aspergillus niger* being the most dominant species isolated across different dilutions. Fungi such as *Aspergillus* were identified based on their distinctive morphological features like aseptate hyphae and conidiophores, which were clearly visible under microscopic examination after lactophenol cotton blue staining. The isolation, subculturing and microscopic observation of fungi can be clearly seen in the following figures.

Fig. 1. Isolation of fungus from peanuts on petri plates

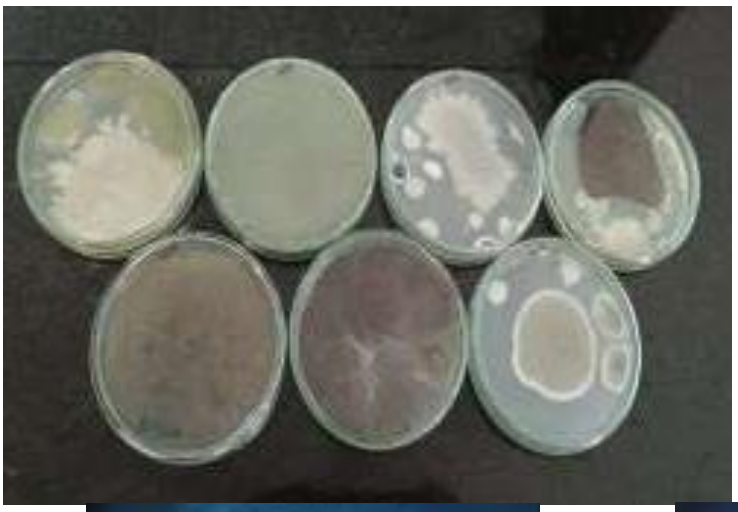

Fig. 2. Subculturing of Fungi

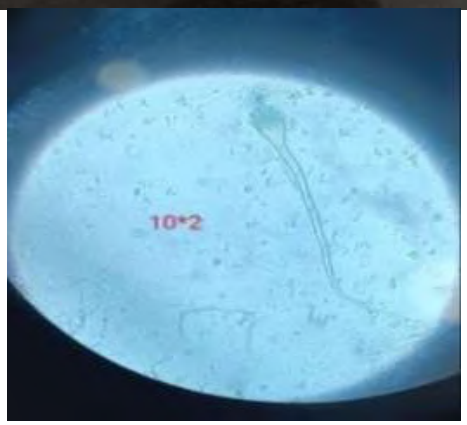

Fig. 3. Complete fungus structure which included hyphae and conidiophores.

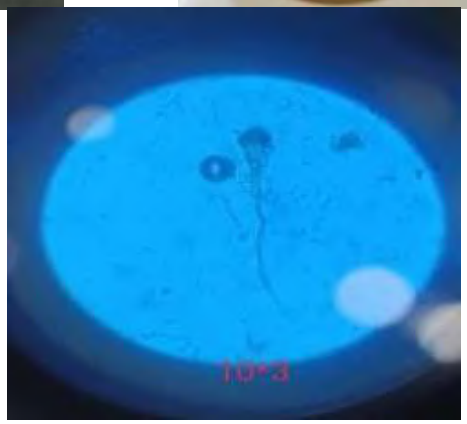

Fig. 4. Sporulating fungal structure

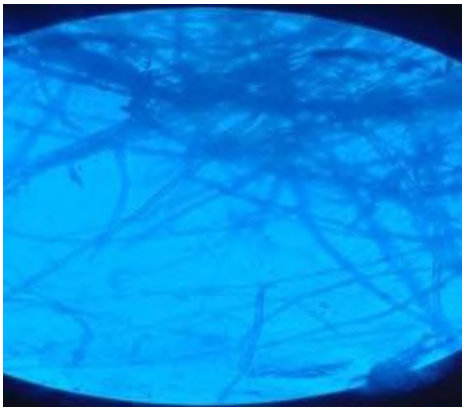

Fig. 5. Complete hyphae structure on different dilutions

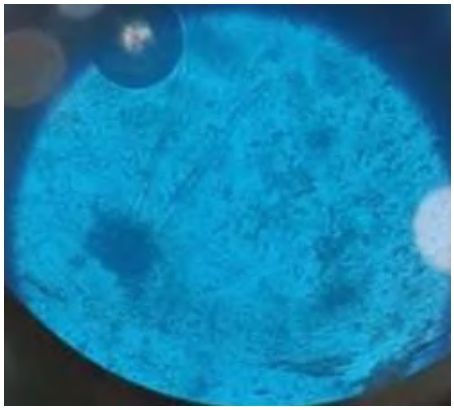

Fig. 6. Fungus obtained from diluted plate

The dilution plates with concentrations from 10^1 to 10^7 showed varying degrees of fungal growth, with the highest concentrations yielding more substantial fungal colonies. Specifically, the plates with lower dilutions (10^1 to 10^5)

presented colonies of *Aspergillus niger* in most cases, characterized by their thick, septate hyphae and conidia formation. On the 10^6 dilutions, no fungal colonies were observed. The revelations of the microscopic observations are:

Table 1. : Morphological revelations by Microscope

Sr. No.	Dilution plate number.	Results	Observation	Strain of fungi obtained
1.	10^1	Positive	Aseptate hyphae, circular conidia	*Aspergillus niger*
2.	10^2	Positive	Aseptate unbranched short hyphae, spherical conidia	*Aspergillus niger*
3.	10^3	Positive	Thin Aseptate unbranched long hyphae, circular spores, sphericalconidia.	*Aspergillus niger*
4.	10^4	Positive	Only hyphae observed due to incomplete growth of conidiophores	-
5.	10^5	Positive	Aseptate thin, unbranched hyphae with small spherical conidia	*Aspergillus niger*
6.	10^6	Negative	No fungus observed	-
7.	10^7	Positive	Solid thin Aseptate hyphae with solid spherical conidia	*Aspergillus niger*

Microscopic examination revealed distinct fungal structures such as conidia and conidiophores, which helped in confirming the genus and species. In addition to the morphological analysis, DNA isolation was performed on the fungal mycelia, and the quality of the DNA was confirmed through gel electrophoresis. The DNA bands observed were sharp and distinct, suggesting successful extraction and good DNA quality suitable for further molecular analysis.

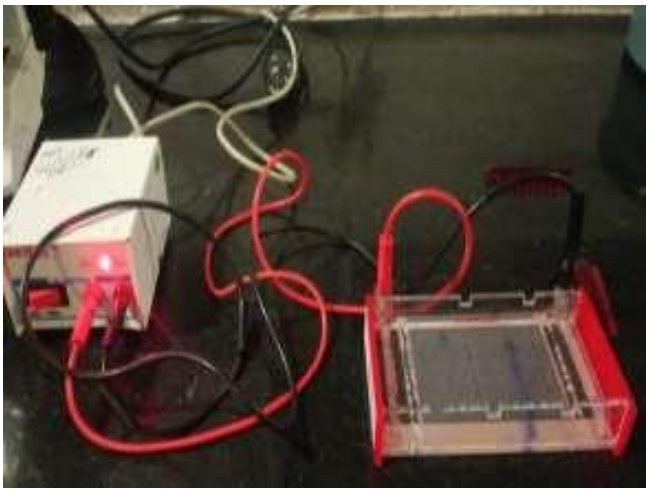

Fig. 7. Gel Electrophoresis Kit

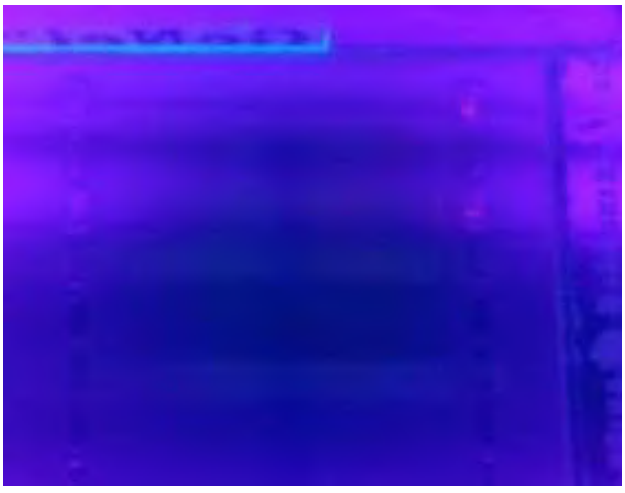

Fig. 8. Agarose gel viewed under UV

6. DISCUSSION AND CONCLUSION:

The results of this study shows that peanut kernels are extremely susceptible to fungal contamination, with *Aspergillus niger* being the most commonly isolated species. The moist conditions in which the samples were stored had a strong influence on fungi growth, allowing fungal colonies to form more easily. The presence of *Aspergillus niger* is especially concerning because it is known to produce mycotoxins, such as aflatoxins, which pose serious health risks to humans and animals when consumed. This is consistent with previous research demonstrating the ability of *Aspergillus* species to thrive in oil-rich seeds in favourable environments.

The morphological characteristics of the isolated fungi, such as septate hyphae and conidia, were consistent with those of *Aspergillus niger*. Lactophenol cotton blue staining allowed for clear visualisation of these structures,

making it easier to identify the fungal species. Subculturing and microscopic analysis confirmed the findings, validating the isolation method used.

In addition to morphological identification, DNA was successfully extracted from isolated fungal colonies and its quality was confirmed using gel electrophoresis. The presence of distinct and sharp DNA bands demonstrated that the isolation and purification process had been successful. This opens the door to further molecular research, such as PCR amplification or sequencing, which could improve the accuracy of fungal identification and provide deeper insights into the genetic diversity of the fungi associated with oil seeds. The results also highlight the importance of preventing fungal contamination in oil seeds, particularly during storage. Moisture is critical in promoting fungal growth, and improper storage conditions can cause significant biodeterioration of oil seeds, lowering their nutritional value and introducing harmful toxins. This has both economic and health implications, especially in areas where peanuts and other oil seeds are staple foods.

Finally, the study successfully isolated and identified Aspergillus niger from peanuts, highlighting the possibility of fungal contamination in oil seeds. The combination of morphological and molecular methods used in this study was found to be effective for fungal identification. Future research could look into molecular approaches for more precise identification, as well as the development of natural fungicides or improved storage techniques to reduce fungal growth. The information gathered here provides valuable insights into the management of fungal contamination in oil seeds, with the goal of ensuring food safety and avoiding economic losses caused by fungal spoilage.

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