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ENZYMATIC PROFILING, ISOLATION, AND IDENTIFICATION OF *ASPERGILLUS NIGER*

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Abstract

A filamentous fungus, *Aspergillus niger*, has tremendous agricultural and industrial importance and was used by many researchers for the production of industrially important enzymes. Many *Aspergilli* were recognized as both human pathogenic and mycotoxin producers too. Even though various *Aspergillus* species have been used widely in research, their taxonomy is unclear due to very close morphological features among their species. In the present study, different *Aspergillus niger* strains were isolated from the decayed fruits and vegetables, and the enzyme profiling for the production of industrially important enzymes like amylase, pectinase, lipase, cellulase, catalase, and urease enzymes was done. Among four different strains isolated, strain number RSS01 was producing, a high yield of pectinase, and on molecular identification, it has been identified as *Aspergillus niger strain RA401* and submitted to Genbank bearing an accession number MN153032. Hence this local strain can be used in the large-scale production of Pectinase enzymes.

Keywords: Morphology, taxonomy, *Aspergillus niger*, enzyme activity, Genbank Accession number MN153032.

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INTRODUCTION:

Aspergillus was a type of mold that was constantly present in the environment. It can sporulate within a few days of germination and can be white, green, yellow, red, or even black. Despite the complexity of the *Aspergillus* taxonomy, the genus can be quickly identified by its distinctive visual characteristics using the various taxonomic keys and guides that are currently available [11]. Aspergillus is a varied group of species, with estimates of its current species count ranging from 260 to 837[8] [21] [11].

Out of many subspecies that make up this genus, a black *aspergillus* called *nigiri* is recognized as important species with biotechnological uses [20]. This prevalent mold, *Aspergillus niger*, was proven to be a great source of numerous bioactive substances, industrial enzymes, and numerous applied research disciplines [14]. *A. niger* is safe to use in the food business [25][19] and hence was used as a microbial factory for the synthesis of industrially important enzymes like β-amylases, cellulases, lactases, invertases, pectinases, and acid proteases. The detrimental effects of the genus *A. niger* on humans are well documented, as well as its good effects [17] It is well known that this fungus damages baked goods, fruits, and vegetables, causing financial losses.

Enzymes are necessary for all living cells and they are proteinous in nature [16]. Microorganisms are cell factories, popular for the production of industrially important enzymes as they are economic, nontoxic, and environmentally friendly [14]. A significant number of microorganisms were responsible for the production of enzymes and were considered to be safe from the biotechnological perspective. Fungi are often used for large-scale production of different enzymes as they were considered as simplest organisms to handle. The use of fungi-produced enzymes is widespread, spanning industries like food, chemicals, medicine, agriculture, and energy [21]. The usage of *Aspergillus* species benefits from traits including the availability of a secretory system, the possibility of genetic manipulation, and high productivity employing various fermentative techniques [14].

There has been extensive research on the potential of Aspergillus species' enzymes for use in creating commercial items [25]. Hence the goal of this investigation Hence, the main goal of this investigation is to identify high-yielding Aspergillus niger strains from deteriorated fruits and vegetables. The enzymes amylases, pectinases, lipases, catalases, and cellulases have very much importance in the food and beverage industry. The Urease enzyme is useful in the wine and brewing industry for preservation.

MATERIALS AND METHODS:

2.1 Isolation and purification of fungal strains: Fruits and vegetables that had deteriorated were gathered from several Warangal, and Hanamkonda locations and kept at a constant temperature of 4^0 C for future usage. Each sample's substrate, sampling date, and sampling location were noted. On a potato dextrose agar medium, black spores of A. niger were isolated and cultivated from the specimens. Spores were aseptically introduced to the PDA medium from infected fruits and vegetables. Petri plates with the inoculum were incubated at 25 ℃ for 3–4 days. Black *Aspergillus* colonies that were actively growing were subcultured on a brand-new PDA medium to purify the cultures. Once more, the inoculation plates were incubated for 6-7 days at 25 °C (until full plate growth). All isolates were preserved for identification and future reference at 4 °C.

2.2 Species identification and differentiation: The isolates obtained after purification were identified by their morphology [19] and were differentiated based on biochemical tests. The Characterization was carried out based on sequencing on the LSU 18S rRNA-based molecular technique, amplified by PCR.

a. Morphological and cultural studies: Based on both physical and cultural findings, a description of each isolate was created. The color, size, and zonation of the colony were visible to the naked eye, whilst a light microscope was used to examine the conidial heads' shape, the presence of exudates, the upstanding and submerged mycelium, the

sclerotia, and the sexual status (if present). Conidia, vesicles, conidiophores, back cells, and striation can all be seen under a microscope in terms of shape, size, and ornamentation [19]. Once authenticated, all isolates were identified by comparing these statistics to published true literature. Creative and microphotography have also been done for report and reference.

b. Biochemical studies: The variability amongst the isolates was once studied on the foundation of an undertaking of catalase, lipase, pectinase, cellulase, amylase, and urease tests as described below.

c. Test for Catalase activity: Based on the breakdown of H_2O_2 into water and oxygen, catalase activity is determined. Using the technique described by Bailey and Scott (1994) [2], the A. niger isolates with Catalase activity were identified. A 3mm disc of fungal isolates that were actively developing was introduced aseptically into a substrate that contained 3% H_2O_2. The appearance of oxygen bubbles surrounding the fungal disc is evidence that the isolate is catalase-active.

d. Test for Cellulase activity: The cellulase-active isolates were identified using the technique described by KC *et a.l* (2008) [12]. Gram's iodine was poured into the 1% Carboxy methyl cellulose (CMC) fungal growth medium. Within 3 to 5 minutes, Gram's iodine created a crisp, identifiable zone surrounding the microbial colonies that produce cellulase by forming a bluish-black complex with cellulose but not with hydrolyzed cellulose.

e. Test for Lipase activity: The isolates with Lipase activity were determined based on the method given by Haba *et al.* (2000) [10]. 1% (w/v) Tween 80 was used as the substrate which was added to the medium with composition 2% (w/v) peptone, 1% (w/v) NaCl, 2% $CaCl_2.2H_2O$ and 2% (w/v) agar and the pH of the medium was adjusted to 6.0. in the center of the Petri plates, an actively growing fungal disc of 3mm was placed and incubated for 48 hours. The activity of Lipase was identified by the opaque halo around the inoculum.

f. Test for amylase activity: The isolates with amylase were identified based on the method given by Behailu Asrat *et al* (2018)[3] The fungal growth medium containing 1% starch agar was inoculated with the 3mm of isolate and allowed to grow for 48 hours, after the growth of the organism the Petri plate was flushed with an iodine solution. If the enzyme is presents it forms a blue around the halo.

g. Pectinase activity test: The isolates with pectinase activity were identified based on the method given by Sudeep *et al* [13]; The fungal growth medium containing 1% (w/v) pectin as substrate, 0.14% (w/v) $(NH_4)_2SO_4$, 0.6% (w/v) K_2HPO_4, 0.30% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4.7H_2O$ was inoculated with actively growing 3mm culture disc and is incubated for 48 hours at 25^{o}C. The fungal isolates utilize the pectin substrate hence the radius of the no-growth region helps to find out the activity of pectinase.

h. Test for Urease activity: The phenol red rapid Urease test given by Finegold and Baron, 1986[6] was used to identify the Urease activity. To the basal PDA growth, medium 0.001% (w/v) phenol red was added aseptically where 3mm fungal inoculum discs were added to the petri plates and were incubated for 48 hours at 25^{o}C. Positive Urease activity is identified by the pink color in the culture.

i. Molecular analysis: For molecular analysis, identification of fungal culture using the D1/D2 region of LSU (Large Subunit: 28S rDNA) based molecular technique was done.

j. Isolation of Fungal DNA: For isolation of fungal DNA, 1.5 ml of Eppendorf tube was filled with 500 µl of PDA medium, inoculated with some hyphal threads, and allowed to grow for 72 hours at 25^{0}C. The pelleted mycelial mat was collected by centrifuging at 13,000 rpm in a cold centrifuge for 5 minutes. The pellet obtained was washed again with 500 µl of TE buffer and centrifuged again to collect the pellet. After TE was decanted, 300 µl of extraction buffer was added (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The mycelia were crushed in a motor pestle and vortexes at 200 rpm for some time. To the above, 150 µl of 3 M sodium acetate, pH 5.2 was added to keep tubes at -20^{0}C for 10 minutes. An equal volume of isopropanol was added to the above and after 5 minutes the precipitated fungal DNA was collected at room temperature. This DNA was washed with 70% cold ethanol, and now the pellet was dried for some time and was resuspended in 50 µl of TE

((10 mM Tris, 0.1 mM EDTA, pH 8)[5].

Experimental method for Molecular technique: The quality of the DNA isolated using the aforementioned technique was evaluated. 1.2% Agarose Gel was used to evaluate the quality. From the plasmid DNA previously obtained, a fragment of the D1/D2 region of the LSU (Large subunit 28S rDNA) gene was amplified by PCR. To get rid of impurities, the PCR amplicon was cleaned. Using the BDT v3.1 Cycle sequencing kit and the ABI 3730xl Genetic Analyzer, a forward and reverse DNA sequencing reaction of a PCR amplicon was performed. Using aligner software, a consensus sequence of the D1/D2 region of the 28S rDNA gene was created using forward and reverse sequence data. BLAST was performed with the nr database of NCBI GenBank database. The phylogenetic tree was constructed based on the maximum identity score for the first ten sequences.

RESULTS AND DISCUSSION

1.1 Identified Isolates: From different deteriorated fruits and vegetables, four strains of A. niger were isolated. The details of these isolates were given in Table 1, where a unique number, which was used throughout this investigation, was assigned to each isolate.

1.2 Morphological Characteristics of the isolates: All the isolates were showing similar microscopic observations. All the isolates were showing black aerial mycelium, white substrate mycelium, spores in glubose, spore surface rough and irregular, and spore mass black shown in Fig. 1A

2. **Activity of Enzymes:** It is generally known that *A. niger* produces a variety of industrial enzymes (KC *et al*). In this work, the capacity to generate four distinct enzymes by *A. niger* isolates from various sources was evaluated. The variation in these enzymes produced by the isolates was employed as a genetic difference indicator (if any).

3. The fungus *A.niger is* widely known for the production of many crucial enzymes [12]. In the present study, the isolated *A. niger* from different sources was tested for the production of enzymes viz; catalases, lipases, pectinases, amylases, cellulases, and ureases. Even though all these isolates showed similar morphological observations under a microscope, their enzyme production profile was seen to be different and presented in Table 2.

The synthesis of the same enzyme by various strains exhibits significant variation, which reveals the genetic diversity of those strains. The findings of this investigation show that all of the isolated strains produced the enzymes catalase and urease. According to a study on catalase by Eda BAYKAL SARI et al. (2020) [22], the growth media affects how well *A. niger* produces this enzyme. Urease enzyme levels were high in the strain identity number RSS01. Cross-referencing the literature revealed that only two of the 13 *A. niger* strains obtained by Ghasemi *et al*. (2004) [9] were exhibiting the greatest levels of Urease production. Strain identity nos. RSS02, RSS03, and RSS04 have moderate and little urease activity. The strains RSS01 and RSS04 have no Lipase activity. RSS02 has high Lipase activity and RSS03 has moderate activity.

According to Ashutosh Nema (2019)[16], the activity of Lipase depends on the substrate and medium of production. RSS01 has medium Amylase activity and RSS04 has poor amylase activity. According to Asrat et al (2018)[3] production of Amylases depends on different parameters during production. RSS01 was showing moderate and RSS04 poor production of Cellulase. According to Sulyman AO *et al* (2020) [24] production of cellulase enzyme depends on optimal conditions like temperature, pH, etc.

Among all the strains isolated, strain identity number RSS01 was showing maximum activity for different enzymes like pectinase, Urease, and moderate activity for catalase this isolate was selected for further molecular identification.

4. **Molecular analysis:** The D1/D2 region of the LSU (Large subunit 28S rDNA) gene sequencing is carried out, and the BLAST was carried out to confirm legitimate identification because it is difficult to identify isolated organisms based just on morphology. The first 10 sequences were chosen based on the greatest identity score, and a phylogenetic tree was built using MEGA software. The phylogenetic tree

is presented in Fig. 2. The strain identity No. RSS01 culture, which was labeled as **Sample** was found to be ***Aspergillus niger strain RA401*** **(GenBank Accession Number: GQ169752.1)** based on nucleotide homology and phylogenetic analysis. The nucleotide sequence of Strain identity no. RSS01 which was identified using the D1/D2 region of LSU (Large subunit 28S rDNA) gene sequencing was submitted to Genbank with Accession number MN153032.

Conclusion:

The objective of this work is to isolate and identify the strain-producing industrially important enzymes. The strain number RSS01 has been identified to produce high levels of Pectinase and Urease. Among these enzymes, pectinase (includes a group of enzymes: Polygalacturonase, pectin esterase, and pectin lyase) has global importance in the food processing industry and other biotechnological importance, as microbial pectinases are eco-friendly natural tools for their production. The enzyme profiling for different isolated strains was performed to identify which isolate has the maximum potency to produce industrially important enzymes. Hence this locally isolated strain can be used for a further study like the production of enzymes and purification of enzymes like Polygalacturonase or pectin lyase or pectin esterase.

Table 1: Information of isolates used in this study

Strain identity No.	Host infected	Contaminated plant/ tissue
RSS01	*Allium sativum*	Bulb
RSS02	*Lycopersicon esculentum*	Fruit
RSS03	*Citrus limon*	Fruit
RSS04	*Malus domestica*	Fruit

Table 2: Various *Aspergillus niger* strains' enzymatic activity

Strain identity No.	Amylase	Lipase	Cellulase	Catalase	Pectinase	Urease
RSS01	++	-	++	++	+++	+++
RSS02	-	+++	-	++	-	+
RSS03	-	++	-	+	-	+
RSS04	+	-	+	+	+	+
Enzymatic activity is represented as No activity '- ', Poor activity '+', Moderate activity '++', High activity'+++'						

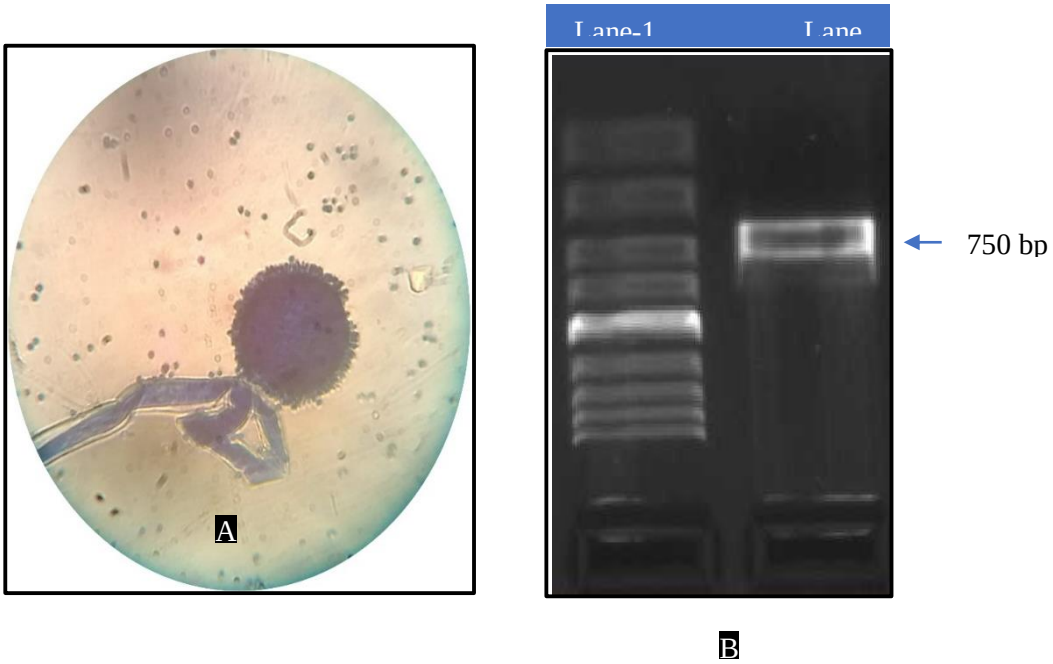

Fig 1: ***A***- *Morphology of Aspergillus niger strain identity number RSS01 under Microscope,* ***B***- Gel electrophoresis image showing an image of the D1/D2 region of large subunit 28S rDNA of RSS01, Lane1: DNA marker, Lane 2: Amplicon band with 750 bp

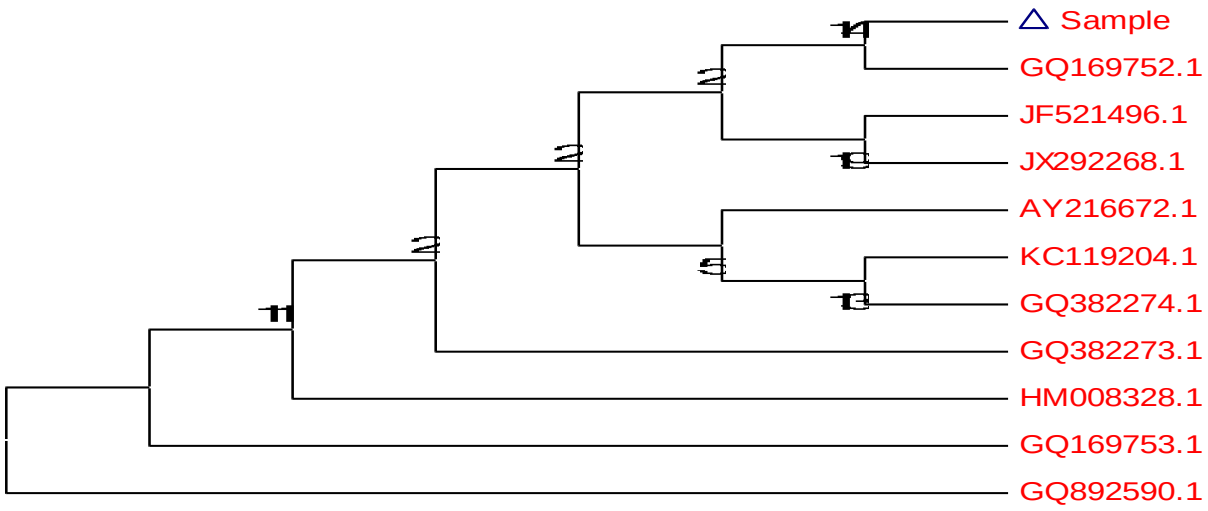

Fig 2. The Strain identity No. RSS01, which was labeled as **Sample** was found to be ***Aspergillus niger strain RA401*** **(GenBank Accession Number: GQ169752.1)** based on nucleotide homology and phylogenetic analysis**.**

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