

Comparative Screening and Integrative Identification of Indigenous, High Yielding, Glutaminase Producing *Aspergillus* Strains Isolated from Spoilt Rice

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ABSTRACT

Fungi, an abundant eukaryote, show diverse characteristics and produce many valuable biomolecules with wide industrial applications, making accurate identification essential in bioprocesses. A combination of morphological, microscopic features and molecular characterization guarantee some confidence in fungal identification compared to the morphological and microscopic features alone. L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is an endopeptidase that specifically catalyses the breakdown of L-glutamine to L-glutamic acid and ammonia. The enzyme can be obtained from many sources but fungal sources prove to be more sustainable. The current study is aimed at unveiling the glutaminase production potentials of strains of *Aspergillus* species isolated from spoilt rice and efficiently identify the best producer using a combination of morphological, microscopy features and molecular identification tools. Spoilt rice samples were inoculated onto modified Czapek Dox (MCD) plates for obtaining isolates and characterizing their morphological features. Glutaminase production potential was evaluated by inoculating the isolates into MCD broth, incubating for 5 days, and measuring absorbance at 540nm. Scanning electron microscopy was employed for microscopic characterization. The DNA of the best producer was extracted using Presto™ Mini gDNA Yeast Kit (Geneaid, GBYB100) and amplified using PCR and sequenced employing Sanger's DNA sequencing. BLAST search was made using the sequences obtained in order to ascertain the identity of the organism at hand, phylogenetic tree was constructed via neighbour joining. A total of seven fungal isolates were obtained from spoilt rice among which isolate SR05 exhibited the highest glutaminase activity (0.544), followed by SR02 (0.503), while SR04 showed the lowest (0.172). The observed differences were not statistically significant (p 0.3220). BLAST analysis identified SR02 and SR05 as *A. oryzae*, whereas SB04 was identified as *A. niger*. In conclusion, isolated and fully characterized indigenous strain of *A. oryzae* isolate possess the highest glutaminase production potential of 0.544 thus, recommended for further study.

Keywords: Fungi Isolation, Molecular Identification, Glutaminase and Production potentials.

INTRODUCTION

Macroscopic and microscopic features are traditionally and commonly employed for fungal identification with Macroscopic characterisation and identification been based on conidia, conidiophore, mycelial colour, colony reverse colour, colony diameter, exudates production, and the formation of sclerotia (Rodrigues et al., 2022), whereas microscopic characterisation is based on vesicle shape, size, and seriation, st pe, hulle cell formation, and cleistothecium (Okayo et al., 2020).

As weighty as it may seem, identifying fungi based on their physical attributes is an antique method of classification (Okayo et al., 2020) because the method is completely deficient of accurateness in discerning

between the various and morphologically diverse fungi due to inter and intra-species similitude (Rodrigues et al., 2022).

The similarities that exist in the genus *Aspergillus* at both the intraspecific and interspecific levels impede the discrimination between the different species using morphology-based systematic identification tools (Okayo et al., 2020) as a result, an erroneous identification is assured which often comes with unpleasant consequences (Rodrigues et al., 2022). In addition, despite the fact that these morphological means of identifications are laborious and time-consuming they require a skilled microbiologist, an expertise that is only acquired and perfect over long period of time between the interval of which many erroneous identifications are made (Godet & Munaut, 2010; Rodrigues et al., 2022).

Conversely, molecular methods, which entail amplifying the different segments of the identified DNA of choice as a target and then sequencing it, are more consistent and thus dependable and thus, can be employed to support morphological keys for proper identification of even the most stringently interrelated members of the *Aspergilli* (Okayo et al., 2020).

Sequel, fungal molecular identification uses the universal barcode of fungus, known as the internal transcribed spacer (ITS) region. When ITS sequencing and visual characteristics (morphological-based identification keys) are combined, *Aspergillus* spp. may be precisely identified down to the species level (Zulkifli & Zakaria, 2017; Hong et al., 2023).

Hoang et al. (2019) described the ITS gene to be a short (500–800 bp), standardised, easily amplified, and divergent DNA fragment between species. Due to its increased reliability over conventional methods, DNA barcoding has lately been officially recognized and accepted as the gold standard for fungal identification (Hong et al., 2023).

There are six important species that made up the *Aspergillus* section *Flavi* and they are both phenotypically and genotypically closely related (Soares et al., 2022). Based on their impact on human health generally, and their ability to produce a potent carcinogenic metabolite, the aflatoxin specifically, they are grouped into two major categories the first been *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. These are the members of the first category that readily produces the aflatoxin (Godet & Munaut, 2010; Soares et al., 2022). While *Aspergillus tamarii*, *Aspergillus oryzae*, and *Aspergillus sojae* do not produce the potent aflatoxin and thus, forms the second group of the section *Flavi* (Godet & Munaut, 2010; Soares et al., 2022).

Enzymes are biocatalysts produced by proliferating cells to bring about explicit biochemical reactions often being part of the metabolic processes of the cells (Hemalatha et al., 2018; da Silver et al., 2022). L-glutaminase (E.C.3.5.1.2) is an amido hydrolase that is known to catalyse the hydrolysis of L-glutamine to glutamic acid and ammonia (El-Fakharany et al., 2019; Jiang et al., 2023). The enzyme L-glutaminase plays a central role in nitrogen metabolism in mammalian cells and has also emerged as an important agent in cancer therapy (Zhu et al., 2023).

Thus, the present study was conducted to identify *Aspergillus* species obtained from spoilt rice using the combination of morphological and molecular characteristics and to explore its Glutaminase production potentials.

METHODOLOGY

Glutaminase Production Potentials

The organisms were isolated from the source sample (spoilt rice) by directly inoculating the sample on modified Czapek Dox's (MCD) media plates and incubated at room temperature for five (5) days. The pure cultures obtained after subculturing were stored in PDA slants for further analysis. The glutaminase production potentials of the isolates obtained was assayed by inoculating the isolates in MCD broth for 5 days and the absorbance was read at 540nm (Hasson et al., 2021) and (Mostafa et al., 2021).

Identification of the fungal isolates

Macroscopic Features

Macroscopic characteristics such as colonial colour and texture, colony diameter, the reverse colour of the colony on the culture plate were observed after the incubation period.

Microscopic Features

Slide culture technique

Slide culture technique was performed using petri dish, glass slide, an SDA agar block, sterile forceps, coverslip and methylene blue. Leica DM 2500 microscope was used and the images obtained were analysed using DinoCapture v2.0.-(Daniel, 2014). Appropriate references were made using mycological identification keys and taxonomic descriptions (Davise, 2002 and Wijedasa and Liyanapathirana, 2012).

Scanning Electron Microscope (SEM)

Scanning electron microscopy (SEM) using (JOEL JSM 6400)–was employed to examine the microscopic characteristics of the fresh 5 days grown isolates. Such important and distinguishing microscopic features for fungal identification as conidiophore, metulae, phialides, conidial size and shape were observed and recorded.

Molecular Identification

DNA extraction and amplification

The DNA of the freshly grown isolates was extracted using Presto™ Mini gDNA Yeast Kit (Geneaid, GBYB100) and the fungal 5.8S ITS gene was amplified using universal primers ITS1 and ITS4 using standard PCR protocols as presented in table 1 and 2 (Raja et al., 2017 and Hoang et al., 2019).

Sequencing

The ITS/5.8S rRNA gene PCR products were purified by the standard PCR clean-up method and subjected to bidirectional sequencing with universal primers M13F (-20) and M13R-pUC (-26) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

A BLAST search using National Center for Biotechnology Information (NCBI), Barcode of Life Database (BOLD) (Hebert et al., 2003) and User-friendly Nordic ITS Ectomycorrhiza Database (UNITE) (Kõljalg et al., 2013) database was made using the sequences obtained to ascertain the identity of the organism, a phylogenetic tree was constructed by neighbour joining technique (Raja et al., 2017) and the sequences obtained were deposited in GenBank for accession numbers acquisition. The ITS gene whose molecular size falls between 550 – 660bp was targeted for the fungal identification. This was achieved with the following set of primers: ITS1; 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4; 5'-TCCTCCGCTTATTGATATGC-3' (Hoang et al., 2019).

Table 1: PCR Conditions for Amplification of ITS Gene of the Isolates

Reaction	Temperature (°C)	Time	No. of Cycles
Initial denaturation	98°C	2 mins	1
Denaturation	98°C	15 secs	25
Annealing	60°C	30 secs	
Extension	72°C	30 secs	
Final extension	72°C	10 minutes	1

Source: Masuo et al., (2004) and Meena et al. (2015).

RESULTS

Glutaminase Production Potentials of the Fungal Isolates

The potential for the spoilt rice isolates to produce glutaminase is revealed by the current investigation. Isolate SR04 had the lowest potential (0.172), whereas isolate SR05 possess the highest potential (0.544). Figure 1 shows the MCD broth tubes used to measure the production potential and Figure 2 shows the outcome. The data are presented as means $\pm$ standard deviations (SD) of three (3) independent replicates ($n = 3$).

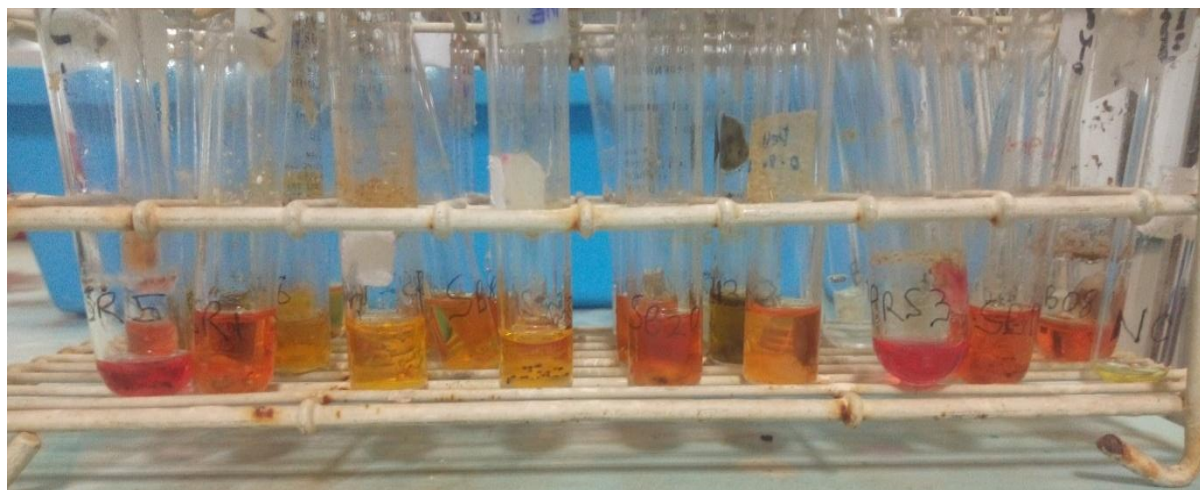


Plate 1: MCD Broth used for Assessing the Glutaminase Production Potentials of the Isolates After Inoculation and Incubation.

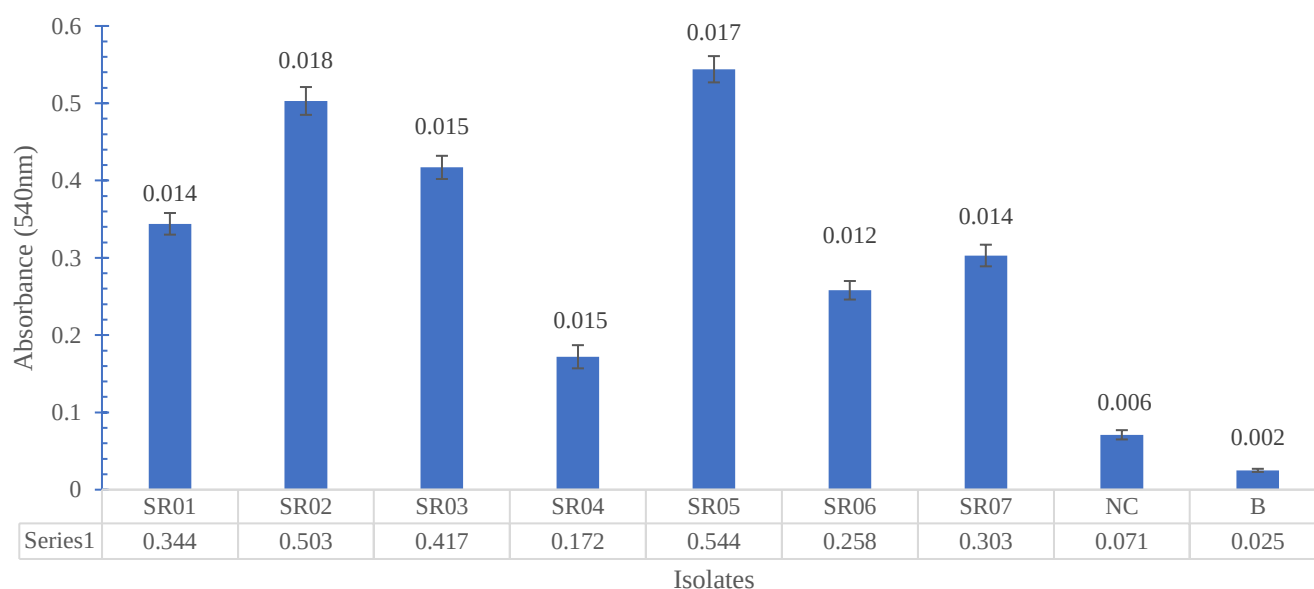


Figure 1: Glutaminase Production Potentials of the Spoilt Rice Fungal Isolates.

Key:

SR 01-07 – Spoilt Rice Isolates 01 – 07.

NC – Negative Control

B – Blank

Macro and Microscopic Characteristics of the Isolates Obtained

Following the cultural and microscopic routines for identification, the most crucial characteristics taken into account for identification according to Larone's medically important fungi, a guide to identification. Figure 3 shows the morphological characteristics of the isolates on MCD plates, and Figure 4 is a SEM micrographic image that clearly illustrates the exclusive microscopic characteristics of the isolates for unassuming identification.

On an MCD plate, the isolate SR05 displays a pale greenish colony that grows radially from the origin of inoculation. The white mycelial pilling up that eventually turns green as growth continues. Figure 3 shows the prospects of the isolates to produce the glutaminase enzyme on the MCD plate (the MCD culture plate turns pink from yellow). The thick and rough conidiophore contains a globose vesicle from which phialides and metulae develops. The conidia are globose and range in diameter from 2 or 3 to a maximum of 7 μ m as portrayed by Figure 4.



Plate 2: Isolate SR05 on MCD Plates In cooperated with Methylene Red Indicator.

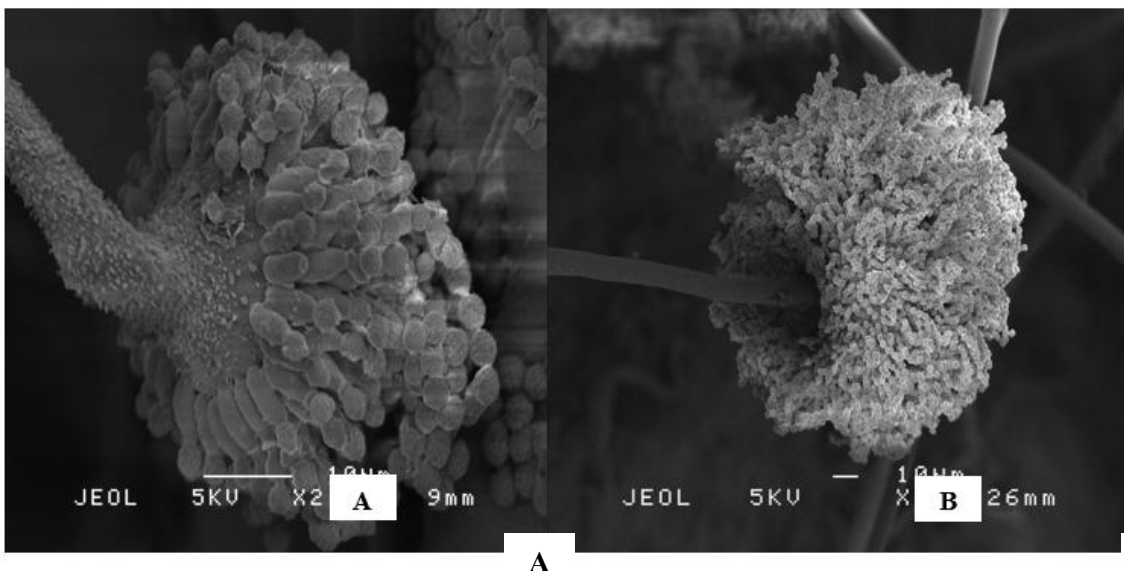


Plate 3: SEM Micrographic Image of (A): *Aspergillus oryzae* (SR05) and (B): *A. niger* (SR04).

Species Distribution and PCR Amplicons of the Isolates

Figure 5 displays the species distribution of the isolates from the sample in question. *Aspergillus oryzae* was the most common, occurring at a rate of 42.86%, while *Aspergillus niger* and *Aspergillus oryzae* were the least common both occurring at 14.29% respectively. While Figure 6 presented the bands of the PCR amplicons of the ITS1-5.8S-ITS2 gene.

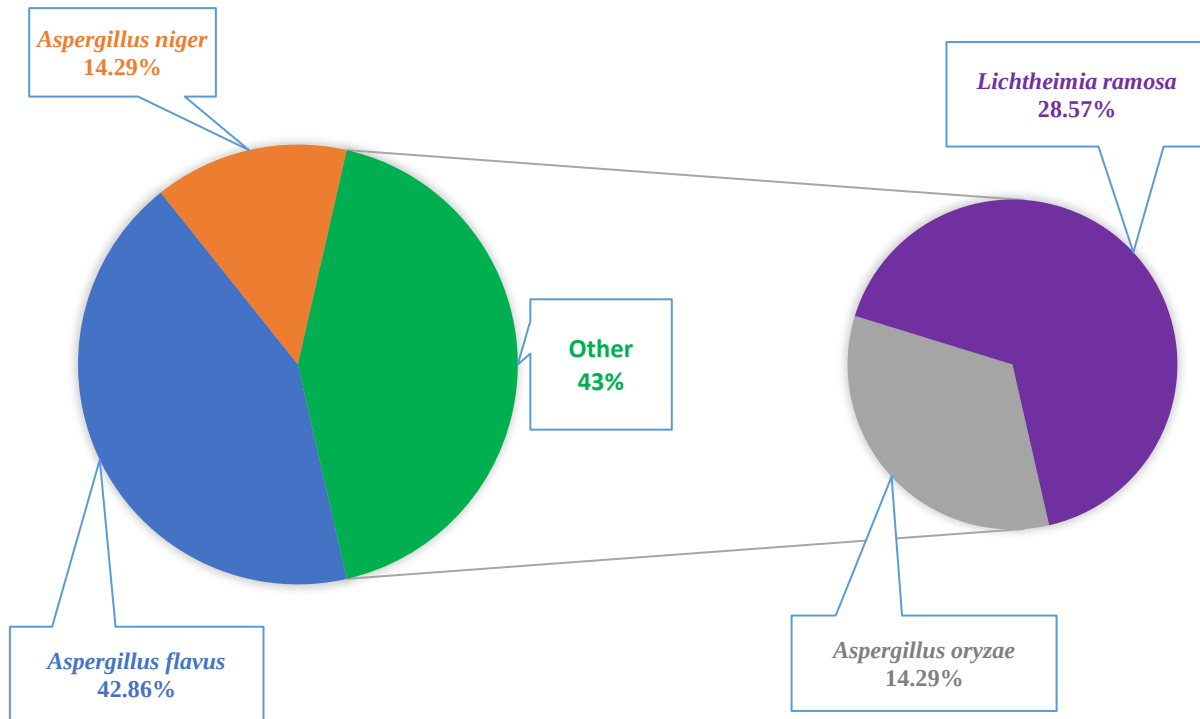


Figure 2: Species distribution of the Isolates from the Spoilt Rice Sample.

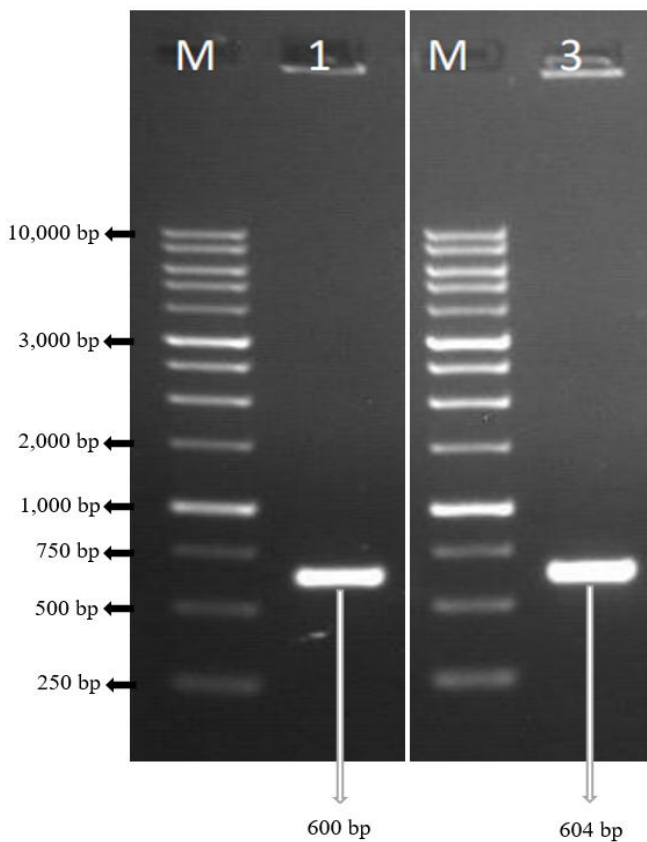


Plate 4: ITS1-5.8S-ITS4 gene PCR Amplicons on 1% TAE Agarose Gel for *A. oryzae* and *A. niger* respectively.

Accession Numbers Acquisition and Phylogenetic Tree

The obtained sequences of the isolates were carefully examined and processed before being deposited and accepted to NCBI. As a result, they were allotted the accession numbers PP430398, PP430399, PP430400,

PP430401, PP430402 and PP430403 for SR01 to SR06 respectively. Maximum Likelihood and the neighbor joining algorithm were used to create the phylogenetic trees of the organisms after BLASTING and aligning the best hits.

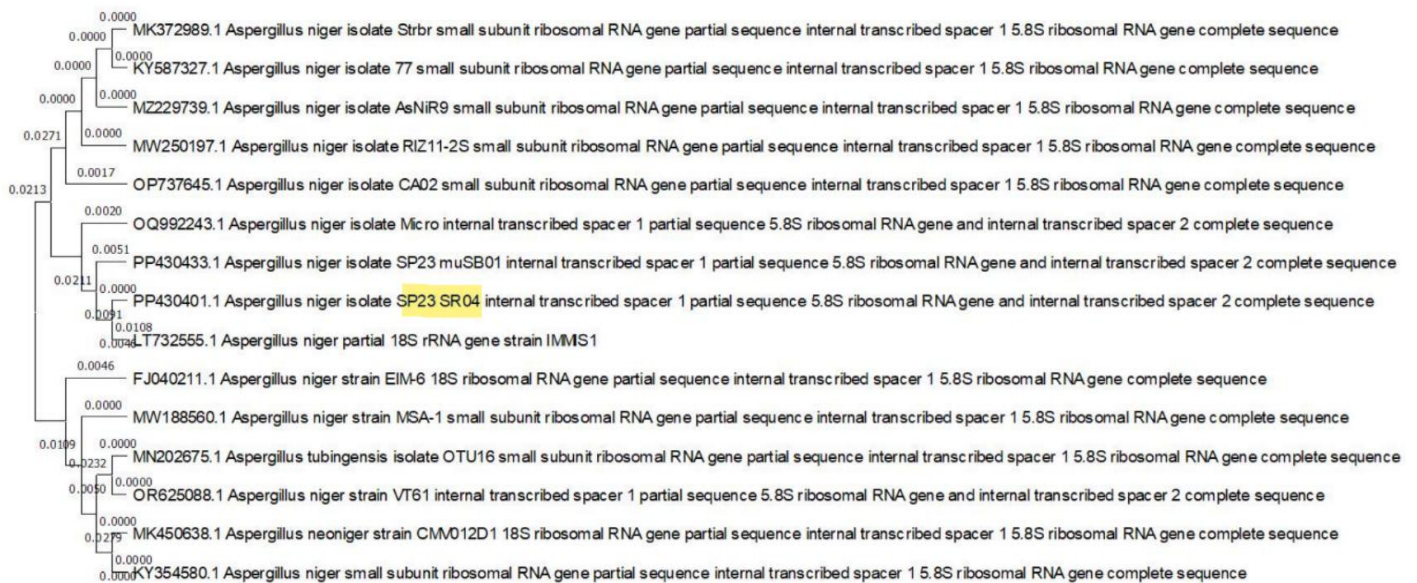


Plate 5: Maximum likelihood Phylogenetic Tree based on the sequences of ITS1-5.8S-ITS4 gene for *A. niger*.

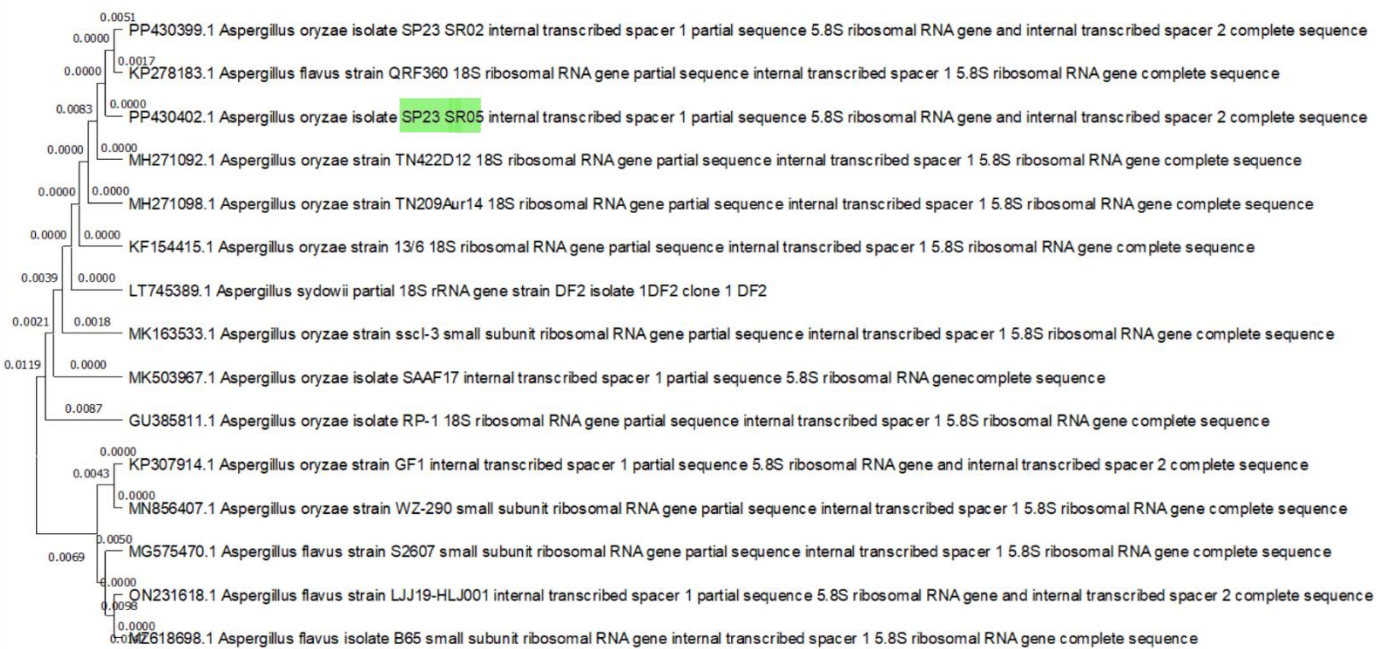


Plate 6: Maximum likelihood Phylogenetic Tree based on the sequences of ITS1-5.8S-ITS4 gene for *A. oryzae*.

DISCUSSION

Glutaminase Production Potentials of the Fungal Isolates

The glutaminase production prospects of a particular isolate can be evaluated by inoculating it into an MCD broth (to which phenol red was incorporated as a pH indicator) and measuring the absorbance of the inoculated MCD broth at 540 nm after incubation. In the present study, the change in the pH of the medium as a result of the glutaminase production makes the yellow MCD broth to turn to pink in colour. The glutaminase production potential of each isolate was evaluated by inoculating it into MCD broth supplemented with phenol red as a pH indicator, followed by measuring absorbance at 540 nm after incubation. Glutaminase activity resulted in a pH shift that changed the broth colour from yellow to pink, with colour intensity corresponding to enzyme

production. Spectrophotometric analysis revealed that SR05 exhibited the highest absorbance (0.552), whereas SR04 showed the lowest (0.102). The isolates SR05 and SR04 had the highest and least absorbance and thus glutaminase production potentials respectively, at 0.552 and 0.102 respectively. With p-value of 0.3220, the differences in the abilities observed were not statistically significant.

The results of this investigation are consistent with those of Hasson et al. 2021. The location, strain, degree of adaptation, and availability of nutrients in the organisms' previous habitat all affect the intensity of the enzyme's production. to which the slight variations between the results of this study and those of Mahajan et al. (2012) can be ascribed to.

The outcomes of this investigation are consistent with those of Hasson et al. (2021) who also found that the colour intensity of the plate varied, depending on the isolate, when phenol red was used in the media, and that the absorbance is in correlation with the enzyme production rate. This variation among isolates is also consistent with the results of Balagurunathan et al. (2010) which demonstrated that the change in colour from yellow to purple-red in media containing bromocresol purple as a pH indicator is caused by the production of glutaminase by actinomycetes isolated from marine sediment.

There are some differences in the absorbance values in our study when compared to Mahajan et al. (2012). Mahajan et al. employed bromothymol blue (BTB) along with phenol red as a pH indicator in the screening protocol, where they observed that the BTB indicator provided a better colour contrast (from yellow for acidic pH to green for neutral pH to blue for alkaline pH) than did phenol red. This is reflected in the difference in the indicator dye, which could be one factor in the discrepancy of absorbance values between the two studies. However, other factors, such as the environment in which the organisms are isolated, the strain-specific characteristics, degree of environmental adaptation, and availability of nutrients in the organism's natural habitat can contribute to the variations in enzyme production intensity shown in our study and in the studies of Mahajan et al. (2012).

Binod et al. (2017) and Amobonye et al. (2019) reviewed glutaminase producing bacteria: These are the genera *Bacillus*, *Pseudomonas* and *Achromobacter*, which have been reported to produce varying quantities of glutaminase, depending on the conditions of fermentation, carbon and nitrogen sources, and the presence of inducers.

The glutaminase production of the isolates studied in our study were (SR01–SR06) generally similar to those reported in the literature, but only a qualitative comparison between the two can be made due to different units used to measure activity (absorbance vs specific activity).

Sathish et al. (2008) demonstrated that the optimization of mixture design led to an increase in the production of glutaminase by *Bacillus* sp. up to 3300 U/gds which is much higher than the unoptimized production rate. This implies that, the media composition and fermentation conditions under our study might be optimised to a larger extent, which may give a higher absorbance value.

The production of extracellular L-glutaminase by *Zygosaccharomyces rouxii* in solid-state fermentation process was reported by Kashyap et al. (2002) and the production of glutaminase was found not only in bacteria but in a wider range of microbial taxa. The variation observed between our isolates (SR01-SR06) is, thus, in agreement with the species and strain specific production patterns which are already well described in the literature.

The observed macro and microscopic features of the isolates obtained in this study matches description by Carmen and Sciortino, (2017) and Davise, (2002). The characteristics observed from SR05 (*A. oryzae*) and SR04 (*A. niger*) closely matches those found in the standards. Following this, it can be concluded that a first and second identification of the organisms is presumed and verified following the molecular identification procedures. As previously explained, an efficient and reliable fungal identification cascade combines the three stages of identification and agree to make the molecular approach as the gold standard for fungal identification.

Species distribution in a given study is very important because it serves as an indicator of species diversity in the habitat from which the organisms were obtained, the habitat's balance and nutrient availability. Together,

these support the organism's proliferation without a significant lag phase when it is introduced into similar, refined or controlled habitat. Figure 5 revealed that *A. oryzae* and *A. niger* had the lowest rate of occurrence (14.29%), followed by *L. ramosa* (28.57%), while *A. flavus* had the highest rate of occurrence (42.86%) in the study.

The ITS gene PCR and sequencing analysis is a widely accepted and reliable tool for fungal identification across many fields of fungal study. The ITS genes of the isolates obtained in the present study were found to be within the range of 600 to 604. This is in strong concordance with Hoang et al. (2019) who described the fungal ITS gene to be within the range of 550-600bp. Isolate SR04 (*A. niger*) whose sequence length was up to 604 was the only exception and that could be ascribed to interspecies and strain variations that can exist even between isolates obtained from the same habitat but different niche or due to mutations.

The Tamura-Nei model (2021) and the Maximum Likelihood technique were used to infer ancestral states. Based on their estimated likelihood at site 1, the tree displays a variety of potential nucleotides (states) for each ancestral node. States with probabilities less than 5% are not included in the set of states at each node, which is arranged from most probable to least likely. By applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances computed using the Tamura-Nei model (Nei and Kumar, 2000), the initial tree for the heuristic search was automatically generated. The evolutionary analyses were carried out with MEGA11.

CONCLUSION

The present study not only disclosed the morphological, cultural, and SEM characteristics essential for identifying the isolates, but also revealed their glutaminase production potentials, with SR05 exhibiting the highest activity (0.544) and SR04 the lowest (0.172). Seven *Aspergillus* isolates from spoilt rice were successfully cultivated, characterized, and sequenced. The sequences were submitted to NCBI GenBank and assigned accession numbers PP430398–PP430403 for SR01–SR06, corresponding to *A. flavus*, *A. oryzae*, *A. flavus*, *A. niger*, and *A. flavus*, respectively. The ITS 5.8S gene lengths of these isolates were 602, 600, 601, 606, and 604 bp, respectively. The evolutionary relationships between the isolates and their closest BLAST matches were further demonstrated through phylogenetic tree analysis. The integration of morphological, cultural, microscopic, and molecular techniques, as adopted in this study, ensured a consistent and reliable identification of the fungal isolates.

It is highly recommended that future research should focus scale-up studies on the enzyme production (optimization of process parameters), enzyme purification, characterization, and a trial in any of the potential application of the enzyme.

Declaration of Conflict of Interests

The authors wish to declare that there is no any conflict of interest.

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