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Isolation and Molecular Identification of Fungi Producing of L-asparaginase Isolated from Makkah Region Soil

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ABSTRACT

The production and extraction of enzyme L-aspiraginase produced by some fungus isolates obtained from different soil samples collected from Makkah region, in Kingdom of Saudi Arabia. There were 20 fungal isolates that were recovered from plant rhizosphere in the south and north of Jeddah city, out of which 8 isolates were able to produce L-asparaginase enzyme in high amounts. The higher producers fungal isolates were identified by using DNA sequencing of ITS-1 and ITS-4 primers and deposited in the GenBank nucleotide sequence database under accession numbers as Nothophomagossy piicola LT592943.1, Aspergillus oryzae XR002735719.1, Mucor circinelloides MF356573.1, Aspergillus oryzae HM064501.1, Aspergillus niger DQ915806.1, Rhizopus oryzae JN003654.1, Penicillium sp. KP256500.1, Actinomucor elegansJN887460.1, and Rhizopus oryzae GU126375.1. Nesslerization test showed the difference between the quantities produced by the strains and it was found that the maximum activity of L-asparaginase on the 5th day, was by the isolate AAB1 (1,99 U/min/ml), followed by AGL3(1.94 U/min/ml) and AGL1(1.90 U/min/ml) after six and five days of incubation, respectively.

Keywords: *Fungi, L-asparaginase, ITS-1, ITS-4*

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INTRODUCTION

L-asparaginase was known as L-asparagine amidohydrolase (EC.3.5.1.1). Microbial L-asparaginase has been widely studied in recent years for its potential applications in the pharmaceutical and food industries (Chand *et al.*, 2020; Muneer *et al.*, 2020). L-asparaginase is a useful anti-leukemia enzyme because of its ability in hydrolyzing L-asparagine into aspartic acid and ammonia (Dias & Sato, 2016). Asn amino acids are a nutrient critical to the spread and metabolic processes of cancer cells, tumors and Asn depletion leading to the death of leukemia cells (Ali *et al.*, 2016). In the proliferation of leukemic cells, L-asparagine is an important amino acid (Narta *et al.*, 2007). L-asparagine is the single known enzyme used for the degradation of amino acid frequently applied in chemotherapy of cancer (Tork *et al.*, 2019; Sargazi & Taghian, 2020). L-asparaginase is considered as a basis of therapy procedures for severe lymphoblastic leukemia and is applied for retardation stimulation and amplification therapy in the total pediatric treatments and most adult management procedures (Pieters *et al.*, 2011).

There is an extensive variety of microorganisms that can yield this enzyme such as algae, yeast, bacterial, actinomycetes, and fungi, with different properties of enzyme of its clinical therapy applications (Yadav *et al.*, 2014), especially bacteria like *Halomonas alkaliantractica* (Alzahrani *et al.*, 2019), *Bacillus licheniformis* (Abdelrazek *et al.*, 2019), *Streptomyces fradiae* NEAE-82 (Soliman *et al.*, 2020), *Sterptomyces* sp. (Alzahrani *et al.*, 2020). Certain L-ASNase producing fungal sp., *Fusarium equiseti* AHMF4 (El-Gendy *et al.*, 2021) *Aspergillus oryzae* CCT 3940 (Benchamin *et al.*, 2019) *Aspergillus terreus* (Hassan *et al.* 2018), *Aspergillus flavus* (Patro *et al.*, 2014) and *Aspergillus terreus* (Farag *et al.*, 2015), *Penicillium* sp. (Cardoso *et al.*, 2020) *Penicillium digitatum* was screened for producing extracellular L-ASNase (Shrivastava *et al.*, 2016). One of the many producing organisms is the yeast *Saccharomyces cerevisiae* and also *S. marcescens* NCIM 2919 (Ghosh *et al.*, 2013).

MATERIALS AND METHODS

Collection of Samples and Purification of isolated fungal strains
Different soil samples were collected from some plants' rhizosphere from Jeddah city from the surface and depth of 10 to 20 cm from different plants rhizosphere, it was serially diluted. Aliquots of 0.1ml of the suspension were evenly spread on Modified Czapek Dox Agar. The plates were incubated at 28C for 72h.

Detection of *L-asparagenase activity of fungal isolates (Qualitative assay)*
Fungal isolates were appropriately screened for L-asparagenase production capacity by a rapid qualitative screening of the plates using (Czapek yeast extract agar medium) and phenol red (2g in 100ml ethanol) with the final concentration of 0.009% just before pouring the plates. phenol red as an indicator was added to the media, pink color zone around the colonies were reflected L-asparagenase activity (Prakasham *et al.*, 2010).

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Quantitative assay of the enzymes by rapid plate method

The potential quantitative estimation of the enzymes in culture filtrate was done by the agar well diffusion method. The fungal Cell-Free Filtrate was harvested by centrifugation at 6000 rpm min-1 for 30 min then filtrate with filter paper. Agar plates were prepared by pouring 20 ml of MCD media into a sterile Petri dish. After plates solidify 7mm wells were punching, using a sterilized cork borer, 100µl of the sample was loaded in the wells, the plates were kept in an upright position at 28±2C° for 24 to 48 hrs (Jain *et al.*, 2012).

Quantitative assay of L- asparaginase by Nesslerization

Enzyme production is detected by determining the NH3 amount emitted from glutamine. 0.5 ml of enzyme preparation was added to 0.5 ml of 0.04M enzyme, 0.5 ml of 0.1 M phosphate buffer (pH 8) and 0.5 ml distilled water. They were then incubated at 37°C for 30 min. 0.5 ml of 1.5 M trichloroacetic acid was added to stop the enzymatic reaction after the incubation period.

Following the addition of trichloroacetic acid, by the addition of the enzyme preparation, the blank was started. 0.1ml of the above mixture was taken and added to distilled water of 3.7 ml. After that, 0.2 ml of Nessler's reagent was also added. Using a visible spectrophotometer, absorbance was measured at 450 nm. Under optimum assay conditions, one international unit of the enzyme (U) was determined the enzyme amount that releases 1 µmol of ammonia from the enzyme. As described by Imada *et al.* (1973), the enzyme yield was expressed as units/ml.

Formula for the calculation of enzyme activity:

$$\text{Enzyme activity (U/ml)} = \frac{\text{µmoles of urea released}}{\text{time of enzyme action} * \text{volume of the enzyme (ml)}} \quad (1)$$

Molecular identification

Genomic DNA extraction

DNA was isolated from fungal isolates using **DNeasy® fungi Mini Kit**

Polymerase chain reaction (PCR)

ITS-u1 and ITS-u4 universal oligonucleotide primers were utilized as described (Cheng *et al.*, 2016). Forward ITS1 (5`TCCGTAGGTGAACCTGCGG3`) and reverse ITS4 (5`TCCTCCGCTTATTGATATGC3`) primers were ordered and synthesized to recover full-length gene.

Database research

The obtained sequencing results were exposed to a BLAST search program (http://www.ddbj.nig.ac.jp/search/blast-j.html) for identification of the similarities with the genes in the gene bank.

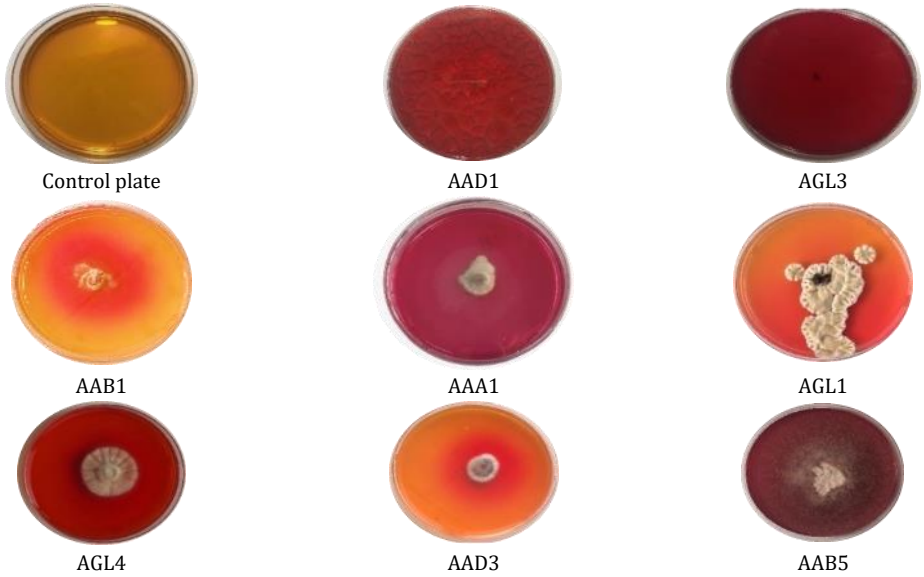
RESULTS AND DISCUSSION

Isolation and screening of fungal strains producing L-asparaginase

Based on serial dilution 20 fungal isolates from different soil samples., fungal isolates AAA1, AAA2, and AAA3 were obtained from north Jeddah, while fungal isolates AAB1, AAB2, AAB3, AAB4, and AAB5 were obtained from the samples from east Jeddah, also the fungal isolates AAD1, AAD2, AAD3, AAD4 and AAd5 isolated from south Jeddah. Three fungal isolates AFA1, AFA2and AFA3 were obtained from the samples collected from Khulais, and four fungal isolates AGL1, AGL2, AGL3and AGL4 were isolated from Alkhumra samples.

Various fungal strains which were isolated from soil samples were preserved on the modified Czapek dox agar (medium) for further studies. Results in **Figure 1** display the collected fungal isolates.

L-asparaginase activity



AAD4

Figure 1. Qualitative assay of L-asparaginase activity of fungal isolates on MCD plates

Qualitative assay of *L- asparaginase activity of fungal isolates*

Fungal isolate's ability for L-asparaginase activity was tested on modified MCD supplemented with L-Asn, as the sole nitrogen source. The initial assay was performed by using phenol red as a pH indicator, Phenol red stain is yellow at acidic pH and changes to pink at alkaline pH, the opposite turn to yellow in the acidic medium. The existence of a pink color region around the colonies on MCD plates with various nitrogen sources was because of the release of the corresponding enzyme.

Quantitative assay of L- asparaginase activity in Cell-Free Filtrate of the selected fungal isolates by diffusion method

The cell-free filtrate of four selected fungi was evaluated L-asparaginase, production by agar well diffusion assay on solid MCD plates with red phenol, after 24h and 48h of incubation, the measurement of the pink zone in millimeters were recorded based on the diffused of L-asparaginase in the agar.

Results in **Figure 2** revealed, the isolates produced different amounts of L-asparaginase after 24h this amounts increased after 48h, the maximum amount of L-asparaginase produced by isolates AAA1, AAB1, AAD3 the diameter of the zone was 58mm, followed by isolate AGL3 the diameter of the zone was 39mm.

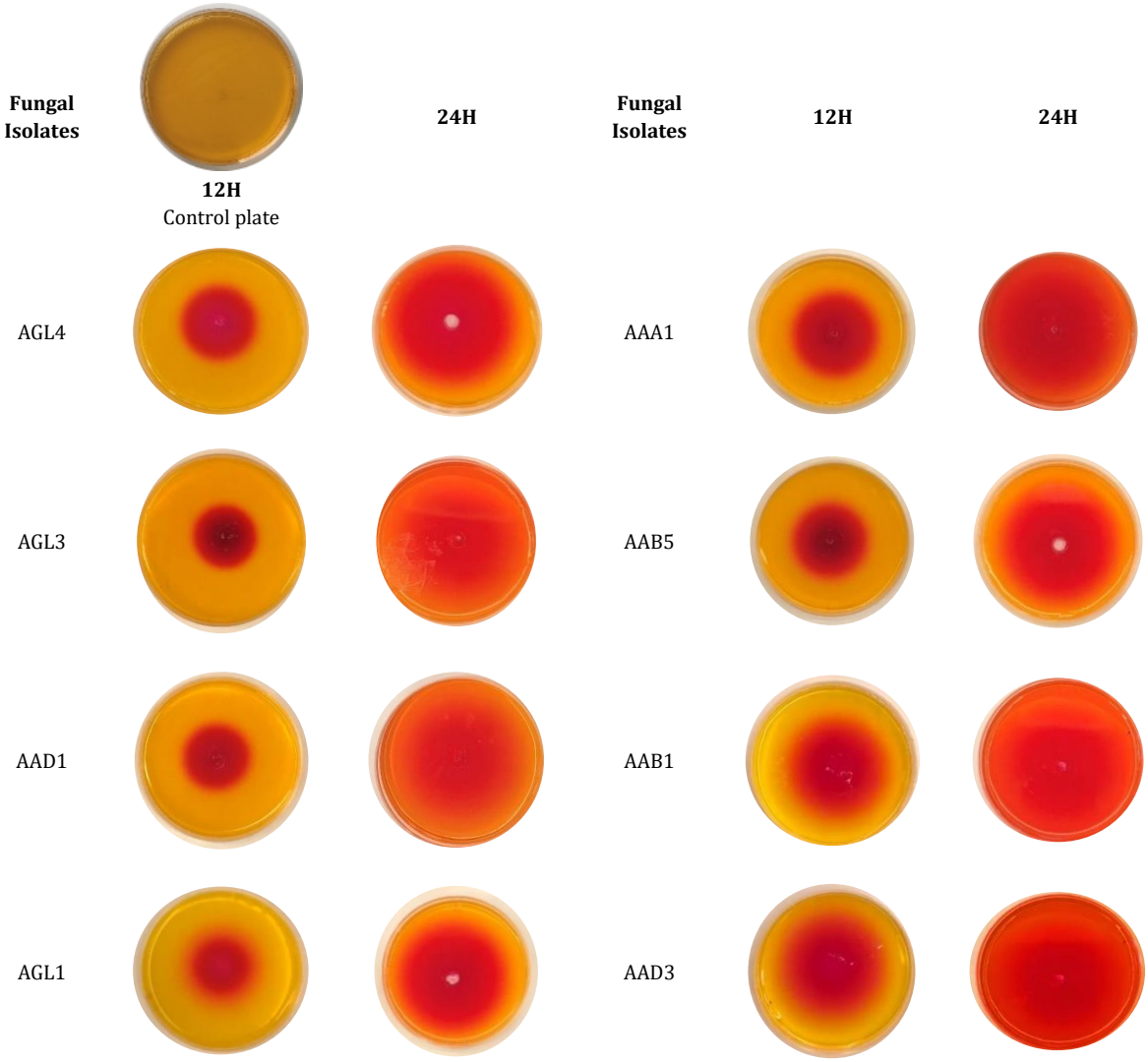


Figure 2. Quantitative assay of L-asparaginase activity in Cell-Free Filtrate of the selected fungal *isolate by diffusion method.*

Quantitative assay of L-asparaginase activity produced by fungal isolates by Nessler's reaction.

The selected fungal isolates were tested quantitatively for L-asparaginase production and assayed for enzyme activity for 7 days. Results in **Figure 3** revealed, the maximum activity of L-asparaginase on the 5th day, was by the isolate AAB1 (1,99 U/min/ml), followed by AGL3(1.94 U/min/ml) and AGL1(1.90 U/min/ml) after six and five days of incubation respectively. The other sex isolates also showed fewer amounts of enzyme in the range of 1.0 to o.6 U/min/ml, the highest amount of each isolate was produced after four days of incubation.

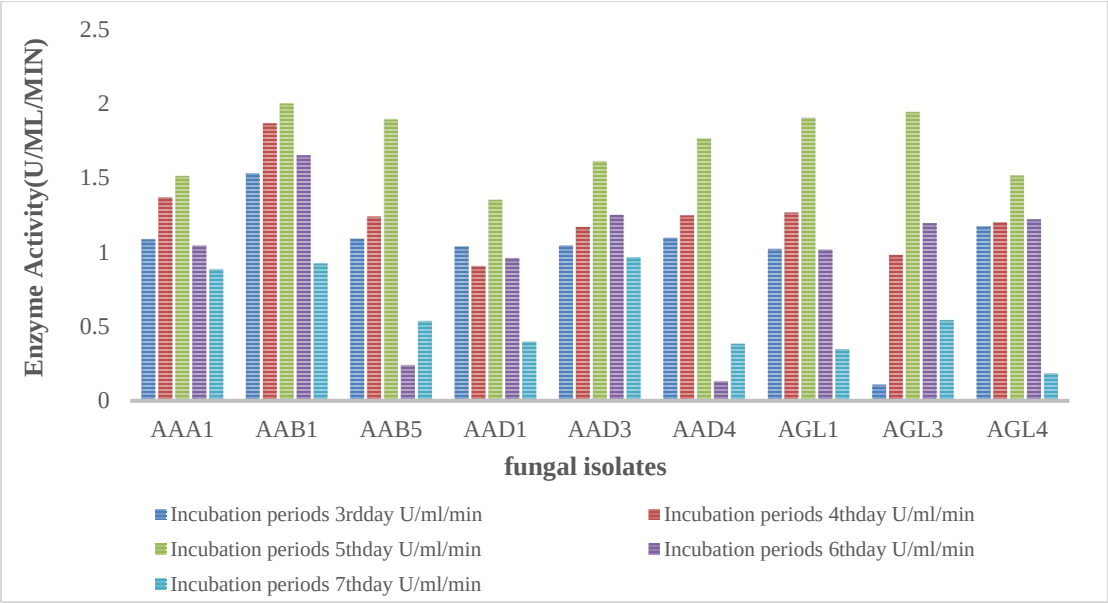


Figure 3. Quantitative assay of L-asparaginase activity produced by the selected fungal isolates by Nessler's reaction

Molecular identification of fungal isolates

The fungal isolates were subjected to molecular analysis and identification using DNA sequencing of ITS-u1 and ITS-u4 primers. First, the 750 bp amplicons were separated on 1% agarose gels to prove their accuracy and specificity. Then, the fragments with the molecular sizes representing the ITS-u1 and ITS-u4 primers were purified for sequencing **Table 1**.

Table 1. DNA sequencing of 18s rRNA using universal primer for fungal isolates

Fungal isolate	Name and Accession of No. of the most related strain in NCBI GenBank		Identity (%)	Coverage (%)
AAA1	LT592943.1	*Nothophomagossypiicola*	100%	100%
AAB1	XR_002735719.1	*Aspergillus oryzae*	91%	100%
AAB5	MF356573.1	*Mucor circinelloides*	100%	100%
AAD1	HM064501.1	*Aspergillus oryzae*	100%	100%
AAD3	DQ915806.1	*Aspergillus niger*	100%	100%
AAD4	JN003654.1	*Rhizopus oryzae*	100%	100%
AGL1	KP256500.1	*Penicillium* sp.	100%	100%
AGL3	JN887460.1	*Actinomucorelegans*	100%	100%
AGL4	GU126375.1	*Rhizopus oryzae*	90%	100%

Natural products have been the source of the most active ingredients of medicines (Harvey, 2008). Microbial screening programs for the production of enzyme began by isolating the microorganisms and then assayed for enzymatic activity by procedures such as the zone of clearance or zone of color change on agar medium supplemented with appropriate substrate (Balagurunathan *et al.*, 2010; Nagaraju & Ram 2019). The identification and examination of filamentous fungi which are able to produce extracellular enzymes with biotechnological capacity are considerably important activities (Zambare, 2010).

The fungal isolates AAA1, AAB1, AAD3 produced different amounts of L-asparaginase after 24h and increased after 48h by isolate AGL3. L-asparaginase's activity in L-asparagine hydrolysis led to aspartic acid and ammonia that transformed red phenol from yellow to pink (El-Naggar & El-Shweihy, 2020). Identical investigations of the production of L-glutaminase by rapid plate assay were found in filamentous fungi by Siddalingeshwara and Lingappa (2010), and with *Fusarium oxysporum* by Hamed and Al-wasify (2016). Eighteen fungal isolates were obtained from Egyptian marine sponge *Aplysinafistularis* L-glutaminase had the ability to produced L-glutaminase (Ahmed *et al.*, 2016).

Our results agree with the studies that discovered that variety of fungi produced the anticancer enzyme L-glutaminase. Still, the exclusive enzyme production in the industry has been carried out mainly by *Aspergillu*s species like *Aspergillus terreus* MTCC 1782 (Varalakshmi & Raju, 2013) may be due to the prevalent nature and non-fastidious nutritional demands of these organisms. Hydrolyses zone surrounding L-ASNase in plate assay was measured to range from 8-13mm for soil bacterial isolates by Devi and Ramanjaneyulu (2016) and (Alzahrani *et al.*, 2019).

About forty-five fungal strains isolated from soil and agricultural remains were examined by using a conventional plate assay process with two indicator stains, phenol red and bromothymol blue (BTB), cleared positive results for L-asparaginase (Doriya & Kumar 2016).

The nine isolates were molecularly analyzed and identified using DNA sequencing of IT1and ITS4 primers. According to Nocker *et al.*, (2004) amplification, ITS1, and ITS4 sequences of fungi can produce 750bp amplicon. The DNA sequences were analyzed using the Blast alignment tools of GenBank. They showed isolates were identified with similarity percentages 99%-100% as: *Nothophomagossy piicola* LT592943.1, *Aspergillus oryzae* XR002735719.1, *Mucor circinelloides* MF356573.1, *Aspergillus oryzae* HM064501.1, *Aspergillus niger* DQ915806.1, *Rhizopus oryzae* JN003654.1, *Penicillium* sp. KP256500.1, *Actinomucor elegans*JN887460.1, and *Rhizopus oryzae* GU126375.1.

About (45%) of fungal isolates showed different activities of L-asparaginase. *Aspergillus oryzae*XR-002735719.1(AAB1) showed the maximum L-asparaginase activity (1, 99 U/min/ml) on the 5th day, Assay L-asparaginase activity from an extracellular source by both methods-(Submerged Culture and Nesslerization reaction) (Dhevagi & Poorani, 2006). The extracellular l-asparaginase production from marine actinomycetes was achieved in both submerged and solid-state fermentation conditions (Basha *et al.*, 2009). Also Oliveira, *et al.*, (2017) used submerged fermentation where *Penicillium chrysogenum* had a greater value of L-asparaginase activity (7.00 U/mL).The enzyme activity produced in liquid media reached 8.3 U min.-1 mL-1 (*Penicillium* sp. T6.2) and 11.4 U min.-1 mL-1 (*Fusarium* sp.) after 72 hours of cultivation in Bacelar-1 medium (Gonçalves *et al.*, 2016). Mishra (2006) reported that *Aspergillus niger* showed maximum activity only after 96 hrs of incubation. Many researchers have cleared that 30°C was the optimum temperature for the production of L-asparaginase by *Penicillium chrysogenum* and *Aspergillus terreus* (Oliveira *et al.*, 2017). The production of Maximum L-asparaginase (126.67U/min/ml) in medium supplemented with asparagine by *Halomonas alkaliantarctica* (Al-Zahrani *et al.*, 2019) and was about 33.59 U/Ml in *Aspergillus* sp. (Doriya & Kumar, 2016). Microorganisms can be easily cultivated and manipulated because they are a good source for enzyme production (Kumar & Sobha, 2012).

CONCLUSION

The current study indicates that the production of L-asparaginase was tested with eight fungal isolates obtained from various soil samples collected from a number of plants in Jeddah, Saudi Arabia. Quantitative and qualitative results showed the high capacity of fungal insulation to produce the enzyme, which is one of the most important enzymes candidates for use in the treatment of cancers. Isolates were molecularly defined by 18S rRNA. It can be concluded from this study that the rooting fungicide activity test has enzyme activity and the extract is expected to be used as therapeutic, industrial and anti-tumor agents**.**

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